Towards the flip of FruR to Er23 via Disulfide Bond Modification

A Senior Thesis

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By

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Abstract

Understanding protein stability and the basis for why a protein folds are very important for the design of biological drugs for treatment of disease. Design of proteins has allowed researchers to discover and analyze many aspects of what it takes to make a protein fold and function under certain conditions, which is one of the key problems in biochemistry. For my project, we are working with two proteins, the DNA binding domain from fructose repressor (FruR or Cra), and a pheromone Er23. These proteins are good sources for mutation analysis because they are small and can be easily manipulated. This aspect will be useful for this project because our main goal is to engineer the FruR sequence to switch to the fold of Er23 or vice versa. We characterize these proteins using NMR, CD, crystallography, and fluorescence. Here we describe a successful variant of FruR that we created by making a mutation at position 25 from an alanine to a tryptophan. This tryptophan probe not only made the protein more thermodynamically stable by a factor of 5 °C, but will allow us to use high-throughput methods and fluorescence in order to observe it's folding and unfolding much more efficiently. We have now turned our attention to expression of the wild type protein Er23, and preliminary results on expression of this pheromone will be described. We will also discuss a strategy to make the smallest number of mutations necessary for Er23 to adopt the FruR fold. We will do this by first engineering the cysteine residues out of Er23, replacing them with Ala-Val mutations. This is because Ala-Val is similar to a cysteine pair in size and we have also had success using this type of mutation with another protein the lab characterizes called Rop. If we are able to completely flip the fold of Er23 into

FruR, this will give us knowledge of what it takes to make a protein fold and what conditions are needed to do so.

Acknowledgements

I would like to thank my advisor, Dr. Tom Magliery for giving me the opportunity to work in his lab as an undergraduate. I would also like to thank Dr. Eva Petrik for training me and for offering continued guidance and support throughout my time in the lab. I would also like to thank Joe Castle for helping me with many experiments for this project. Lastly, I would like to thank my committee members Dr. Jane Jackman and Dr. Gregory Booton as well as all members of the Magliery Lab.

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Fields of Study

Major Field: Biochemistry

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Chapter 1

Introduction

1.1 Protein stability

Protein stability is one of the most complex problems in biochemistry today. The tertiary structure of a protein cannot currently be predicted from the primary structure. Two of the first people to bring this problem to the forefront of biochemistry were Christian Anfinsen and Cyrus Levinthal. Anfinsen made his discoveries in 1960 when he worked on ribonuclease which contains eight sulfhydryl groups causing many possible arrangements with four disulfide bonds. His main interest was in protein folding and finding out how the enzyme folded into its conformational structure based on its primary sequence. Anfinsen believed that certain interactions must guide the formation of the correct disulfide bonds[1]. He later concluded that the most stable conformation of any protein is the native molecule. This conformation is made stable with thermodynamics and correct pairing of disulfide bonds[1].

Through Levinthal's research he was able to conclude something called the Levinthal Paradox. This paradox states that because there are so many possible protein conformations and folded states, it would be impossible to predict by exploring each and every fold using a computer program[20]. This paradox explains why protein stability and predicting the most stable conformation of a certain protein is so difficult.

Other scientists have concluded that the folding of a protein can be defined as a funnel with an infinite number of possible outcomes and conformations ultimately leading to the most thermodynamically stable form. A goal of protein design is to dissect the balance between local and long-range interactions that stabilize a particular fold in

preference to the many conformational possibilities available to a given sequence. The results of protein conformations and folding predictions provide knowledge into how these forces are controlled in both protein structure and with the misfolding phenomena [4]. Although this statement is true, we have not found a way of computationally predicting this most stable conformation and these findings explain why this prediction is so difficult to make.

1.2 Computational and experimental methods

There are two ways in which one can try to answer this complex problem which include computational and experimental methods. My project has included both methods in order to find out as much as possible about this issue. The idea of switching folds between two completely different proteins is a complex one which will allow us to discover more about their structure. Philip Bryan and John Orban have based their research around this computational theory and found that proteins which switch folds are very rare, but they provide useful information of the evolution of protein folding [2].When doing research with many different proteins they realized that three themes were similar among all flipping folds, the structural transitions are in a state with diminished stability; flipping fold requires the flexibility of certain areas of both proteins; there is a possibility of increased stability in an alternate folded state which can lead to new functions[2]. The basic conclusion of their research was that with the existence of protein sequences that create different folds when switching from one native structured protein to another provides questions for understanding how the primary amino acid sequence determines

structure[2]. Overall, it still remains a mystery which proteins can be flipped and how many different conformations are possible based on the primary structure.

Working in collaboration with Ron Elber and his lab they were able to come up with a computational idea in which the fold of one protein, FruR or also called Catabolite Activator Protein (Cra), can be flipped or switched into the fold of a completely different protein analytically, pheromone Er23. There are few (around 2-5) foldable sequences which experimentally can be flipped to a completely different fold with a minor modification in the sequence[3]. This computational analysis helps to predict protein design and switches from which FruR was predicted to switch to Er23 after 50 rounds of mutations to the sequence. To further prove this computational model we set out to experimentally test this *in vivo* and see if this switch between two different structures is actually possible between Er23 and FruR. Figure 1.1 shows the structure of these two proteins and how similar they are to each other.

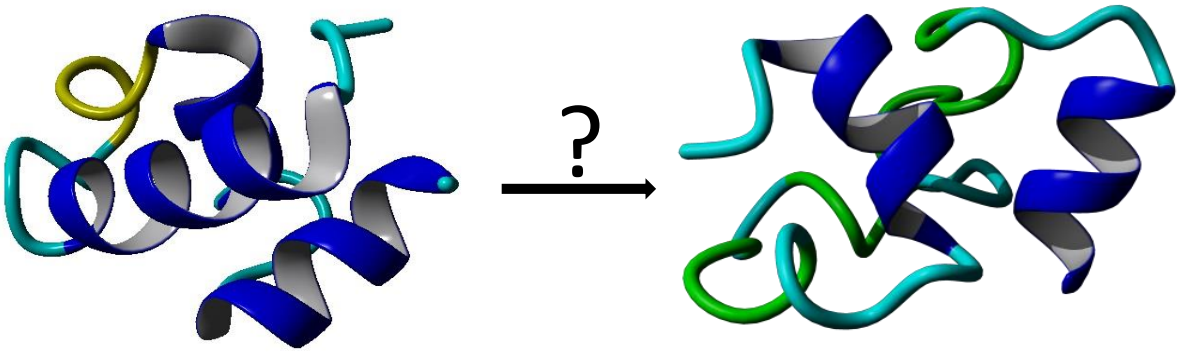


Figure 1.1: The structure on the left, DNA binding domain of FruR 1HA8 and on the right pheromone Er23 1UXC protein shown with alpha helices blue.

1.3: Fructose repressor protein (FruR)

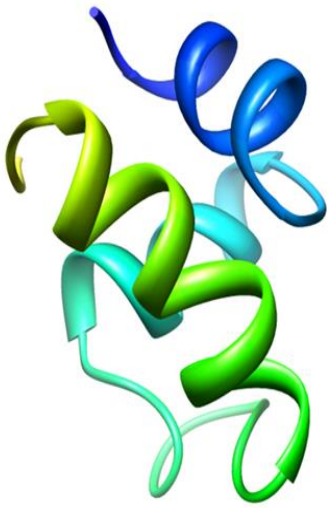


Figure 1.2: crystal structure of FruR Protein (made in Yasara)

The Catabolite Activator protein, also known as Fructose Repressor (FruR) is an *Escherichia coli* transcriptional regulator that belongs to the LacI DNA-binding protein family. By using ^{1}H and ^{15}N NMR spectroscopy, the three-dimensional solution structure of the FruR N-terminal DNA-binding domain has been determined consisting of 57 amino acid residues[6]. The DNA binding domain of this protein is of interest to us because it folds independently into a small globule which can easily be studied by computationally and by biophysical characterization. Previous work has been done on FruR by Venuka Durani[25] and Eva Petrik including a complete Alanine scan of the protein to discover which residues of FruR are of particular importance for its folding into a stable conformation.

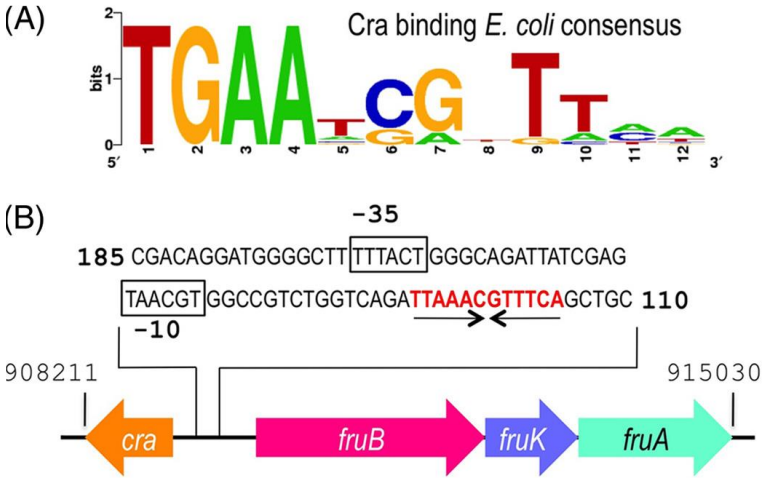


***Figure 1.3: E. coli* FruR binding consensus sequence and regulatory region of the fructose operon of *P. putida*. (Figure from reference 7)**

Figure 1.3 shows the portion of FruR which has the main function of regulating when the binding site is turned on or off. The N-terminal, 60 amino acid fragment of FruR is the DNA-binding domain, while the C-terminal 300 amino acid fragment of the protein remains tetrameric and displays inducer-binding properties and subunit interactions[9]. The N-terminal fragment is small and displays a helix-turn-helix motif similar to the lac-repressor.

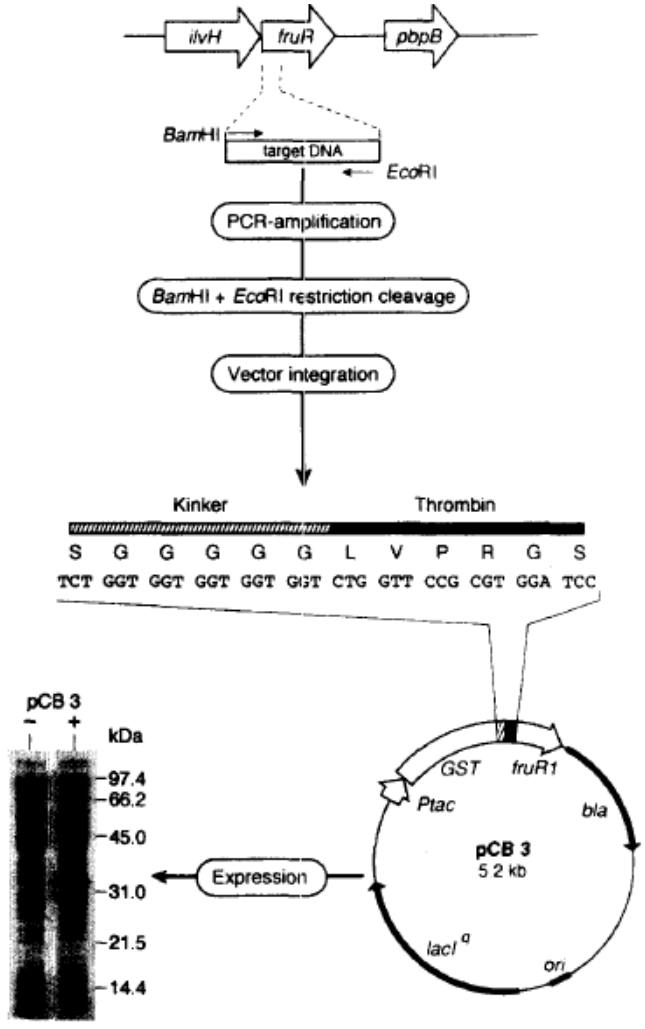


Figure 1.4: Expression scheme of DNA-binding domain of Fructose Repressor protein taken from reference 9.

After the protein was successfully cloned and expressed, with the method for expression shown in Figure 1.4 above, it was purified and then the structure was extensively characterized using proton NMR. They concluded that the FruR regulator is a multi-

domain protein with a structural conformation similar to that of the LacI repressor protein[9].

Earlier studies were done on the FruR proteins in *E. coli* and *S. typhimurium* by Max Chavarria and Cesar Santiago where it was suggested that the regulator recognizes an incorrect DNA sequence and then binds to that incorrect sequence[7]. The crystal structures are shown in Figure 1.5 below.

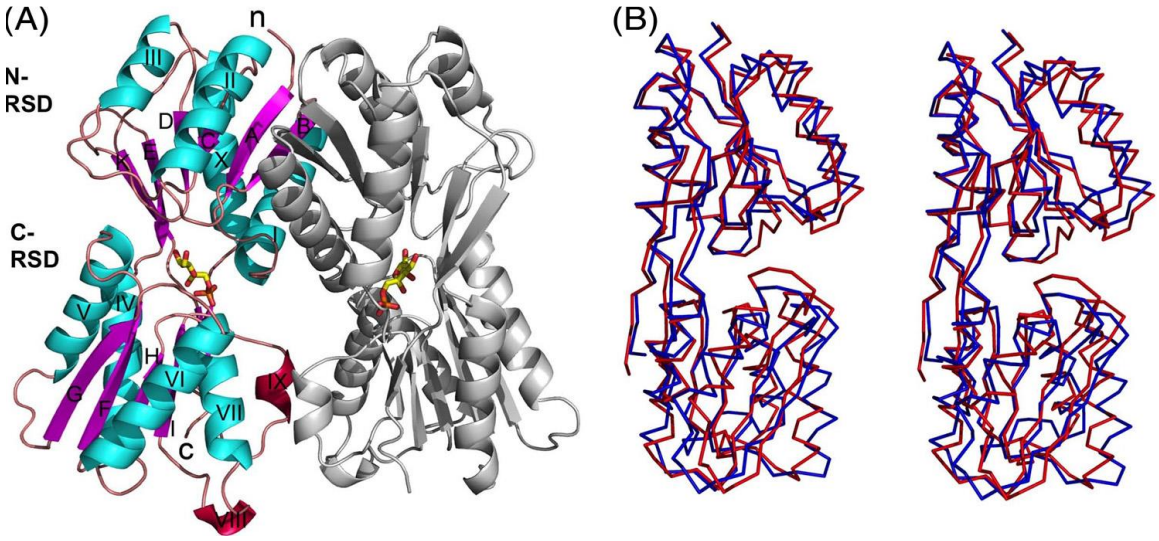


Figure 1.5: Crystal structure of FruR in complex with Fructose-1-Phosphate. (Figure taken from reference 7)

Another study of FruR protein was done by Dayanidhi Sarkar and his lab to provide information about the adaptation of *E. coli* due to the FruR knockout gene[8]. Their studies mainly consist of doing research to find more about the metabolic pathways which depend greatly on carbon sources. Overall, they found that the mutants which had FruR knocked out of the sequence resulted in the lowest yield of activation of the glycolysis pathway. However, the glucose consumption rate stayed the same overall by their studies of this mutant[8]. This analysis explains the importance of FruR for the binding of DNA in *E. coli* and for activation of the glycolysis pathway.

1.4 Pheromone Er23

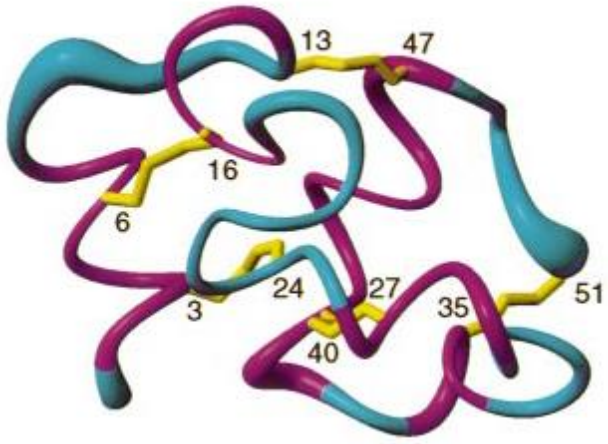


Figure 1.6: NMR Structure of pheromone Er23 (from reference 11)

Pheromone Er23 comes from the protozoan *Euplotes raikovi* and belongs to a group of pheromones which are all purified from the same protozoa[7]. It is a protein with 51 amino acid residues and contains three alpha helices, similar to FruR. The main difference between the two proteins is that Er23 contains ten cysteine residues, making five disulfide bridges. This protein is also quite small which makes it easy to manipulate for characterization and analysis. These five disulfide bridges cause this protein to have a very compacted core. Er23 is unique among presently known Er-pheromones with respect to size and number of disulfide bonds, and the three-dimensional structure[10].

So far only the NMR structure of Er23 has been solved together with the other members of this pheromone family but the protein has not been characterized yet. So, this project will not only allow us to deduce more about the stability problem, but also will give us insight into the properties of Er23 and its pheromone relatives.

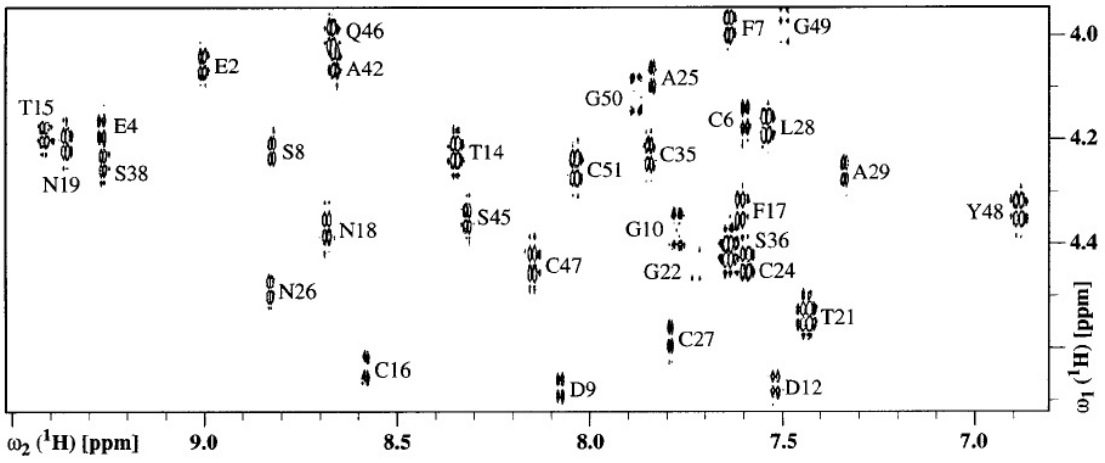


Figure 1.7: [1H, 1H]-2QF-COSY NMR of Er23 from reference 11.

One study of Er23 provided NMR data of the protein shown in Figure 1.7 above and based on these data the structure has been solved[11].

These pheromones are all small proteins of 38 to 51 amino acids with a common architecture based on a bundle of three-helices fastened together by three conserved disulfide bonds. It was also found that their main functions in *E.coli* are prototypic autocrine growth and paracrine mating signals[12]. However, because all of these pheromones contain disulfide bridges, including Er23, this makes expressing and purifying these proteins will potentially be very difficult. Because of this fact previously mentioned, purification was done by extraction from *Euplotes raikovi*[12].

1.5 Successful Expression and Purification of Disulfide Bond heavy proteins

When we decided to express and purify Er23 using *E. coli* cells instead of *Euplotes raikovi* which we knew would be a challenge since this is something which has never been done previously. All previous work done on Er23 was done by extracting the protein from these protozoa. Therefore, we needed to design a complete expression strategy before we could characterize this protein. Under physiological conditions, there

are many reasons why expressing and purifying disulfide-heavy proteins will be especially difficult. For example, the *E. coli* cytoplasm is in a reduced state which disfavors the formation of stable disulfide bonds in proteins and the redox potential for the formation of the disulfide bonds is very low[14]. This doesn't provide a driving force for the formation of stable disulfides which are in active conformation. Also, there are no enzymes available in the cytoplasm of *E. coli* that can catalyze protein thiol-oxidation[14]. Because disulfide bond formation is mediated by thioredoxins, Paul Bessette and his lab asked if increasing the expression of these would enhance disulfide bond formation in the protein. Their experiments found that this was in fact true. They also found that the oxidation of proteins in the periplasm by DsbA may be detrimental for the folding of those proteins containing multiple disulfides[14].

Another study was tested in Michael Reinwarth's lab where the question was asked whether oxidative folding of cysteine-knot proteins or "knottins" would be helpful for forming the correct disulfide bridges[15]. There are specific conditions needed for the formation of correct disulfide bonds. These may include: thiol protection, polymer-supported oxidation, and isosteric disulfide replacements in the presence of redox-favored species, or possibly the deletion of a disulfide bond which is not absolutely necessary for folding[15]. They found that oxidative folding of cysteine-heavy peptides is still very challenging because very specific folding conditions are needed to achieve the most stable conformation of the protein[15].

We also want to figure out more about the stabilization factors disulfide bonds contribute to proteins. We also wanted to deduce whether expressing and purifying "disulfide-heavy" proteins is difficult. They are very important for the overall stability

and structure of the protein in most cases. In a study done by Arnold Mcaulel and his lab they analyzed the effect of disulfide bonds on the stability of antibodies. They found that the disulfide bond could not be formed efficiently by unfolding and refolding the protein without use of redox reagents. In addition, it was difficult to fully reduce the disulfide bond using DTT without partially unfolding the protein first with guanidinium chloride. These observations suggested that the stable structure of folded methane is necessary to prevent the action of the redox agents on the hydrophobic core[16].

1.6 High Throughput Methods for characterization

In order for a more quick and easy way of characterization of the mutants of both Er23 and FruR a high throughput method was needed in order to be as efficient as possible. There are many different high throughput methods which have been studied in Thomas Magliery's lab including intrinsic fluorescence, H/D exchange, cysteine reactivity, aggregation and hydrophobic dye binding, some of which are depicted in Figure 1.8 below[17].

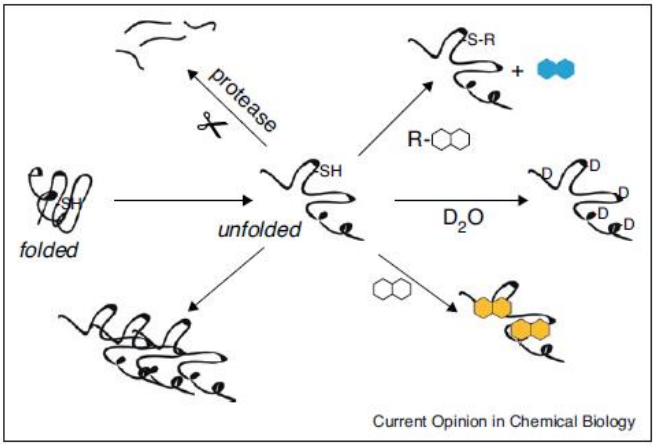


Figure 1.8: Principles of screening for protein stability using high-throughput methods. (Figure from reference 17)

All of these methods have positive and negative characteristics. However, monitoring the denatured and natured states of proteins is most easily seen using fluorescence by aromatic amino acid residues and this is the method we chose to use to monitor FruR and Er23 during our mutation analysis. This fluorescence method is useful because aromatic amino acid residues are fluorescent due to their conjugation containing many double bonds within their structures. Tryptophan gives the strongest fluorescent excitation over the weaker tyrosine and phenylalanine[26]. Once an aromatic amino acid was successfully introduced into the sequences of each protein while maintaining the native structure of the protein, this method could be successfully utilized.

1.7 Constructing cysteine-free proteins

The main difference between Er23 and FruR is that Er23 contains five disulfide bridges while FruR has none. We decided to begin with sequentially removing the cysteines from Er23 or introducing them into FruR to eventually flip to fold of Er23 into FruR or vice versa. Not only did we want to remove the cysteines to start making the sequence of Er23 look more similar to the sequence of FruR, but also because proteins containing cysteine residues, especially many, are very hard to express and purify so removing them would make working with this protein much easier. Some previous studies of Cys-free proteins have been done, specifically on a well-characterized protein in the Magliery lab called Rop. They found that cysteine residues can complicate the folding and storage of proteins due to misfolding of disulfide bonds or oxidation of residues that are normally reduced in their native conformation[18].

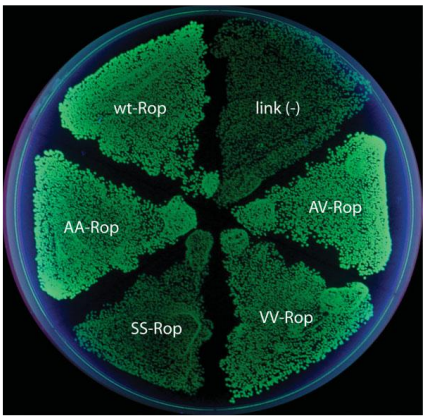


Figure 1.9: In vivo activity of the cysteine variants of Rop from reference 18.

In order to remove the cysteine residues, they had to be replaced with something that would be similar enough to allow the protein to still fold into its native structure. Many different mutations were made that were thought to be similar enough to a disulfide bond to preserve the native structure of the protein. A study was done of these mutants by GFP screening where the more active the GFP the more expression of the ColE1 plasmid where the expression and control of this plasmid is the main function of Rop shown above in Figure 1.9. They found that both the Ala/Ala and Val/Val variants were indeed active. However, only the Ala/Ala variant could be expressed sufficiently under the conditions used[18]. The Ala/Val mutation was the most successful of all the other variants because it allowed for the least destabilization of the protein and made it much easier to work with than the wild-type protein containing disulfide bridges[18] Because this method of mutating out the disulfides and replacing them with Ala/Val potentially creates a protein which is much easier to express and manipulate we decided to use this replacement to start manipulating Er23. Based on these specific findings, our idea was to replace the disulfide bonds in Er23 to both Ala/Val and Val/Ala mutations to see which disulfide bonds (if any) are essential for the correct folding and the stability of Er23. These findings will form the basis of the next step which is modifying the sequence of Er23 to attempt the fold switch between Er23 and FruR.

Chapter 2

Objectives

2.1 Synthesis and characterization of FruR Trp variant

First, as previously discussed, FruR will be expressed containing a fluorescent tryptophan residue in order to use high-throughput methods for studying the stability effects of the mutations. A successful mutation will need to be made where the Trp residue is within the hydrophobic core while still maintaining the WT structure and stability of the protein. Once this has been done, it will make the screening of the states between FruR and Er23 much easier.

2.2 Synthesis and characterization of WT Er23

To characterize and study Er23 in depth, we will need to first successfully express the WT Er23 protein. This will be done using previously used Origami DE3 cells containing the Er23-pHLIC plasmid made by Venuka Durani[25] of Magliery lab. Once successfully expressed, we will characterize WT using CD thermal melts, NMR, and crystallography.

2.3 Synthesis and characterization of Er23 Variants

Once we have obtained more information about the wild type Er23 and FruR, we will then turn our attention to making mutations in Er23 to eventually flip the folding from Er23 to FruR. Our first intuition is to start replacing the cysteine residues selected in Er23 to an already successful disulfide bond replacement of Ala/Val or Val/Ala. This is

because the Er23 contains disulfide bonds within its structure while FruR has no disulfide bonds making this the main difference between the two protein structures.

Chapter 3

Materials and Methods

3.1 Synthesis of WT FruR and Trp Mutants

Gene Construction

Methods of overlap PCR were used for reassembly, and the reactions were performed on a Flexigene thermal cycler (Techne Inc). The WT FruR gene was synthesized from six oligonucleotides (shown in 3.1). The tryptophan variant genes of FruR were also synthesized using these six primers and specific primers containing the specific mutations (primers shown in table 3.2). The genes were synthesized using two separate PCR reactions, the first using the respective six primers, and the second using the forward and reverse primers. Amplification reactions were carried out in a total volume of 50 µL with an annealing temperature of 55 °C for 20 cycles using Pfu polymerase.

FW Primer	ATTATATACCATGGCGAAAATTTATATTTCCAGGGCAGTAGTGGCATGAAACTG
Rev Primer	ATTATAATGGATCCTTATCGCAGACCCGCCGCCAC
Primer 1	GGCAGTAGTGGCATGAAACTGGATGAAATTGCACGCCTTGCTGGAGTGAG
Primer 2	CGTTAATCACATAGCTAGCGGTGGTGCGGCTCACTCCAGCAAGGCGTGCA
Primer 3	CACCGCTAGCTATGTGATTAACGGCAAAGCGAAACAGTACCGAGTGTCTG
Primer 4	CAGCCATCACTTTTTCCACGGTTTTATCAGACACTCGGTACTGTTTCGC
Primer 5	CCGTGGAAAAAGTGATGGCTGTGGTGAGAGAACATAACTATCATCC
Primer 6	TCGCAGACCCGCCGCCACCGCATTTGGATGATAGTTATGTTCTCTCACC

Table 3.1: Primers used to construct WT FruR

I6W-1	GGCAGTAGTGGCATGAAACTGGATGAATGGGCACGCCTTGCTGGAGTGAG
I6W-2	CGTTAATCACATAGCTAGCGGTGGTGCGGCTCACTCCAGCAAGGCGTGCC
V12W-1	GGCAGTAGTGGCATGAAACTGGATGAAATTGCACGCCTTGCTGGATGGAG
V12W-2	CGTTAATCACATAGCTAGCGGTGGTGCGGCTCCATCCAGCAAGGCGTGCA
V38W-4	CAGCCATCCATTTTTCCACGGTTTTATCAGACACTCGGTACTGTTTCGC
V38W-5	CCGTGGAAAAATGGATGGCTGTGGTGAGAGAACATAACTATCATCC
M39W-4	CAGCCCACACTTTTTCCACGGTTTTATCAGACACTCGGTACTGTTTCGC
M39W-5	CCGTGGAAAAAGTGTGGGCTGTGGTGAGAGAACATAACTATCATCC
A25W-3	CACCGCTAGCTATGTGATTAACGGCAAATGGAAACAGTACCGAGTGTCTG
A25W-4	CAGCCATCACTTTTTCCACGGTTTTATCAGACACTCGGTACTGTTTCCA
Y19W-2	CGTTAATCACCCAGCTAGCGGTGGTGCGGCTCACTCCAGCAAGGCGTGCA
Y19W-3	CACCGCTAGCTGGGTGATTAACGGCAAAGCGAAACAGTACCGAGTGTCTG
Y28W-3	CACCGCTAGCTATGTGATTAACGGCAAAGCGAAACAGTGGCGAGTGTCTG
Y28W-4	CAGCCATCACTTTTTCCACGGTTTTATCAGACACTCGCCACTGTTTCGC

Table 3.2: Primers used to construct FruR Trp variants

Cloning

Each of these contained an Nco*I* and Bam*HI* restriction site used for digestion at 37 °C for 2 hours. These inserts were then ligated with pHLIC vector (constructed by Venuka Durani, Brandon Sullivan and Thomas Magliery)[21] which was also digested using Nco*I* and Bam*HI* sites at 37 °C for 2 hours in a total volume of 50 µL. These digested vectors were then combined with the insert in a ligation reaction in a total volume of 10 µL which took place overnight at 16 °C. The now complete plasmid now

containing the gene insert was transformed into electrocompetent DH10B cells which were plated on LB agar containing 50 µg/µL ampicillin and were incubated at 37 °C overnight. Colonies were then picked and the DNA was extracted using Qiagen miniprep protocol. The DNA was then sequenced by Genewiz.

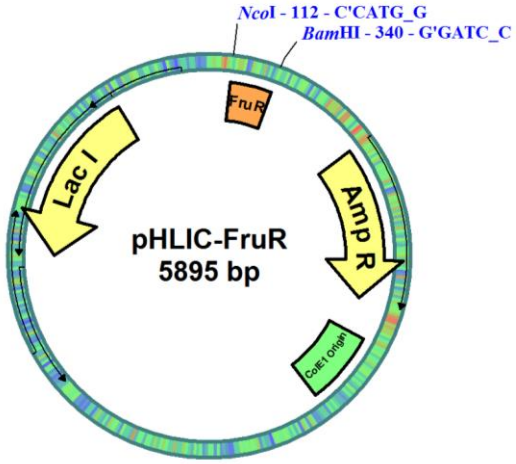


Figure 3.1: FruR cloning vector

Expression and Protein Purification

FruR WT and variants were then transformed into electrocompetant DE3 BL21 cells which were then plated on LB agar containing 50 µg/µL ampicillin resistant antibiotics and were incubated at 37 °C overnight. 1L cultures were grown at 30 °C in 2YT media for 4 hours or until reaching OD_{600}=0.8. The cultures were then induced with 0.1 mM IPTG during the log growth phase and expression occurred for another 6 hours at 37 °C. The cells were then centrifuged and resuspended in 15 mL of lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 8). The cells were then lysed by emulsification. The cell lysate was then extracted from the pellet through centrifugation and was then mixed with 1 mL Ni-NTA slurry for one hour at 4 °C. The mixture was

poured through a pre-fritted column, and the flow-through was allowed to go to waste. The protein was then washed with 12 mL of wash buffer (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, pH 8) and eluted with 1.5 mL of elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 8). Then 1.5 mL of CD buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.2) was added to dilute the protein solution. TEV protease (0.5 mg) was added twice along with 14 µL DTT and allowed to incubate at room temperature overnight. The solution was desalted with PD-10 columns (GE Amersham) into CD buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.2) and mixed with 1 mL Ni-NTA slurry (Qiagen) at 4 °C for a minimum of one hour. The solution was added to a pre-fritted column again and cleaved protein was collected. Protein was then concentrated using an Amicon Ultra-4 Centrifugal filter (Millipore) for future characterization.

CD Spectroscopy

Both variants and WT FruR were analyzed on a Jasco J-185 CD Spectrometer at 50 µM concentrations, determined by UV absorption at 280 nm. Wavelength scans of the variants and WT were taken from 190-260 nm. Thermal denaturation scans were also taken, measuring 1 °C/min from 25-90 °C at 222 nm. Scans were acquired by Eva Petrik.

Fluorimetry

WT FruR and Trp variant fluorescence were measured at 100 µM using an Olis DM45 fluorimeter. Samples were excited at 295 nm and data was analyzed using Olis

Spectral Works Software. The data was collected using absorbance scans from 310-410 nm taken by Dr. Eva Petrik.

M5 Experiment

Protein analysis was done using small volumes of protein (20 µL) in a 384 well plate. Data was collected usisng absorbance scans from 300-400 nm taken by Dr. Eva Petrik.

3.2: Synthesis of WT Er23 and Cys-free variants

Gene Construction

Methods of overlap PCR were used for reassembly, and the reactions were performed on a Flexigene thermal cycler (Techne Inc). The WT Er23 gene was synthesized four primers (shown in Table 3.3). Variant genes were synthesized using four new primers containing their specific mutations which contain the positions replaced with either alanine or valine (primers shown in table 3.4). The genes were synthesized using two separate reactions, the first, a Klenow reaction using the respective two primers, and the second, a PCR reaction using the forward and reverse primers. Amplification reactions were carried out in a total volume of 50 µL at an annealing temperature of 55 °C for 20 cycles using Pfu polymerase. Each of these contained an NcoI and BamHI restriction site used for digestion at 37 °C for 2 hours.

Er23 WT 1	GGCGAATGCGAACAGTGCTTTAGCGATGGCGGCGATTGCACGACCTG CTTCAATAATGGCACCGGCCCATGCGCGAATTGCCTGGCGGGCTATCC GGCGGGG
Er23 WT 2	GCAACCTCCATAGCACTGGCTCAGAAACGCGGTACAGTCGCTATTGCT GCACCCCGCCGGATAGCCCGCCAGGCAATTCGCGCATGGGCCGGTGC C
Er23 FW	ATATATGGTACCGGCGAATGCGAACAGTGC

Er23 Rev	AATATACTCGAGTTAGCAACCTCCATAGCACTG

Table 3.3: Primers for synthesis WT Er23

Er23 AV 6-16 1	GGCGAATGCGAACAGGCGTTTAGCGATGGCGGCGATTGCACGACC GTGTTCAATAATGGCACCGGCCCATGCGCGAATTGCCTGGCGGGCT ATCCGGCGG
Er23 VA 6-16 1	GGCGAATGCGAACAGGTGTTTAGCGATGGCGGCGATTGCACGACCG CGTTCAATAATGGCACCGGCCCATGCGCGAATTGCCTGGCGGGCTA TCCGGCGG
Er23 6-16 2	GCAACCACCGTAGCATTGACTCAAAAAAGCGGTACAGTCAGAATTG CTGCAGCCCGCCGGATAGCCCGCCAGGCAATTCGCGCATGGGCC
Er23 6-16 FW	ATTATATACCATGGCGAAAATTTATATGGCGAATGCGAACAG
Er23 6-16 Rev	ATTATAATGGATCCTTAGCAACCACCGTAGCATTG
Er23 3-24 AV 1	GGCGAAGCTGAACAGTGCTTTAGCGATGGCGGCGATTGCACGACCT GCTTCAATAATGGCACCGGCCCAGTGGCGAATTGCCTGGCGGGCTA TCCGGCGG
Er23 3-24 AV 2	GCAACCTCCATAGCACTGGCTCAGAAAAGCGGTACAGTCACTATTG CTGCACCCCGCCGGATAGCCCGCCAGGCAATTCGCCACTGGGCCGG TGCC
Er23 3-24 AV FW	ATTATATACCATGGCGAAAATTTATATTTCCAGGGCGAAGCTGAAC AG
Er23 3-24 VA 1	GGCGAAGTTGAACAGTGCTTTAGCGATGGCGGCGATTGCACGACCT GCTTCAATAATGGCACCGGCCCAGCGGCGAATTGCCTGGCGGGCTA TCCGGCGG
Er23 3-24 VA 2	GCAACCTCCATAGCACTGGCTCAGAAAAGCGGTACAGTCACTATTG CTGCACCCCGCCGGATAGCCCGCCAGGCAATTCGCCGCTGGGCCGG TGCC
Er23 3-24 VA FW	ATTATATACCATGGCGAAAATTTATATTTCCAGGGCGAAGTTGAAC AG
Er23 3-24 Rev	ATTATAATGGATCCTTAGCAACCTCCATAGCACTG

Table 3.4: Primers for synthesis Er23 Cys-free variants

Cloning

The cloning of these Er23 WT and Cys-free variants were completed using the same protocol as FruR. We also constructed a pHLIC-preMBP plasmid for cloning to afford periplasmic expression.

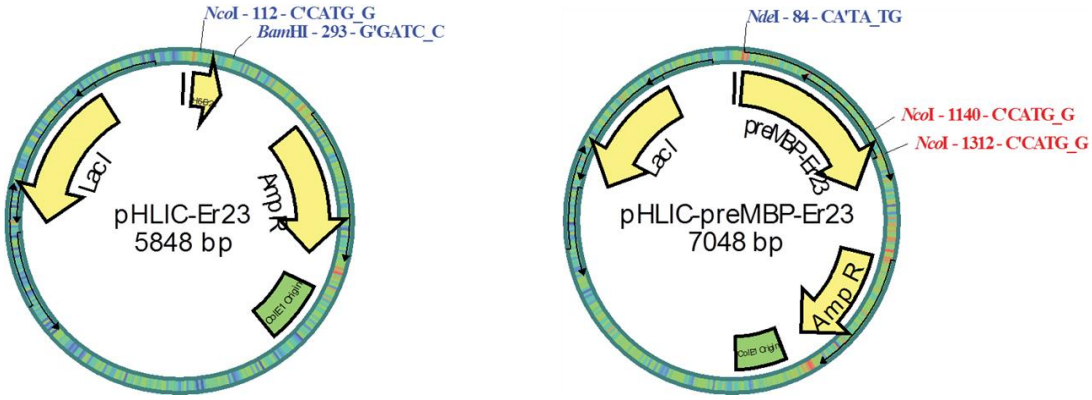


Figure 3.2: Vector maps for WT Er23 cloning

Protein Expression and Purification

Er23 WT and variants were expressed by the same protocol as FruR. However WT Er23 was previously cloned into Origami B: *F⁻ompT hsdS$_B$(r_B^- m_B^-) gal dcm lacY1 ahpC (DE3) gor522: Tn10 trxB pLysS (Cam^R, Kan^R, Tet^R)* from EMD. Once induced during the log growth phase, the expression occurred overnight at 37 °C. The protein was then purified by the same method as FruR described above except the solution was desalted and imidazole removed by dialysis into CD buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.2). Protein was then concentrated using an Amicon Ultra-4 Centrifugal filter (Millipore) for future characterization.

CD Spectroscopy

Both variants and WT Er23 were analyzed on a Jasco J-185 CD Spectrometer. Further protein stability studies were conducted by 7 M Guanidine and 9 M Urea melts at various concentrations with and without the addition of the disulfide bond reducer TCEP. Scans were taken by Eva Petrik.

HSQC-NMR

Each variant was synthesized using the previously mentioned protocol, but in minimal media (0.5 M Na citrate, trace metal solution, 1 M $MgSO_4$, 40% Glucose, 10

mg/ml thiamine, dH_2O) instead of rich 2YT. ^{15}N-labeled NH_4Cl was used as the nitrogen source, and variants were exchanged into CD buffer by dialysis. Samples were concentrated to 1 mg/mL and mixed with 10% D_2O directly before experiments were conducted. The ^{1}H, ^{15}N-HSQC spectra was obtained from a 600 MHz Bruker NMR with inverse probe using standard Lewis E. Kay pulse sequences at 25 °C. The actual scans were acquired by Dr. Chunhua Yuan.

Crystallography

Er23 Cys-free variants crystals were obtained from sitting drop trays at 9 °C with a 1:1 protein to mother liquor ratio. Protein samples were dialyzed into CD buffer and 10 mM HEPES buffer.

Ellman Reaction Experiment

DTNB stock solution (19 mL KPO_4, 159.4 mg DTNB, 60 mg Sodium Bicarbonate, pH 7) was prepared along with 1M Tris (pH 8). The DTNB working reagent was prepared by adding 50 µL DTNB solution, 100 µL Tris solution, and 840 µL water with the final volume equaling 1000 µL with a 10 µL sample. The solution was mixed and cuvette was placed into UV spectrophotometer and a background scan was taken using the solution as the background. 10 µL of the sample solution was added to 990 µL of DTNB reagent. The solution was mixed well and incubated at room temperature for 5 min. The optical absorbance was then measured at 595 nm. The molarity of (-SH) groups was calculated by finding the absorbance and dividing by 13600 $M^{-1}cm^{-1}$ (extinction coefficient of reagent).

Chapter 4

Results

4.1: Synthesis and Characterization of FruR Trp Variant

When deciding on what mutations to make to the WT FruR protein, we consulted with previous work done by Venuka Durani[25] and Eva Petrik. They had previously done a complete alanine scan of the entire sequence of FruR. This alanine scan would allow us to deduce which specific amino acids were absolutely necessary for the specific folding of FruR. Those residues which were important were not chosen to mutate. We then also consulted the LacI-GalR family of proteins from which FruR is a part of. We looked at those sequences which were similar to FruR, but which had a tryptophan variant integrated within the sequence. These residues which seem to be buried in the hydrophobic core of the protein; 39W and 25W were chosen based on this family sequence alignment and showed us where Trp can be found in other members of this LacI family.

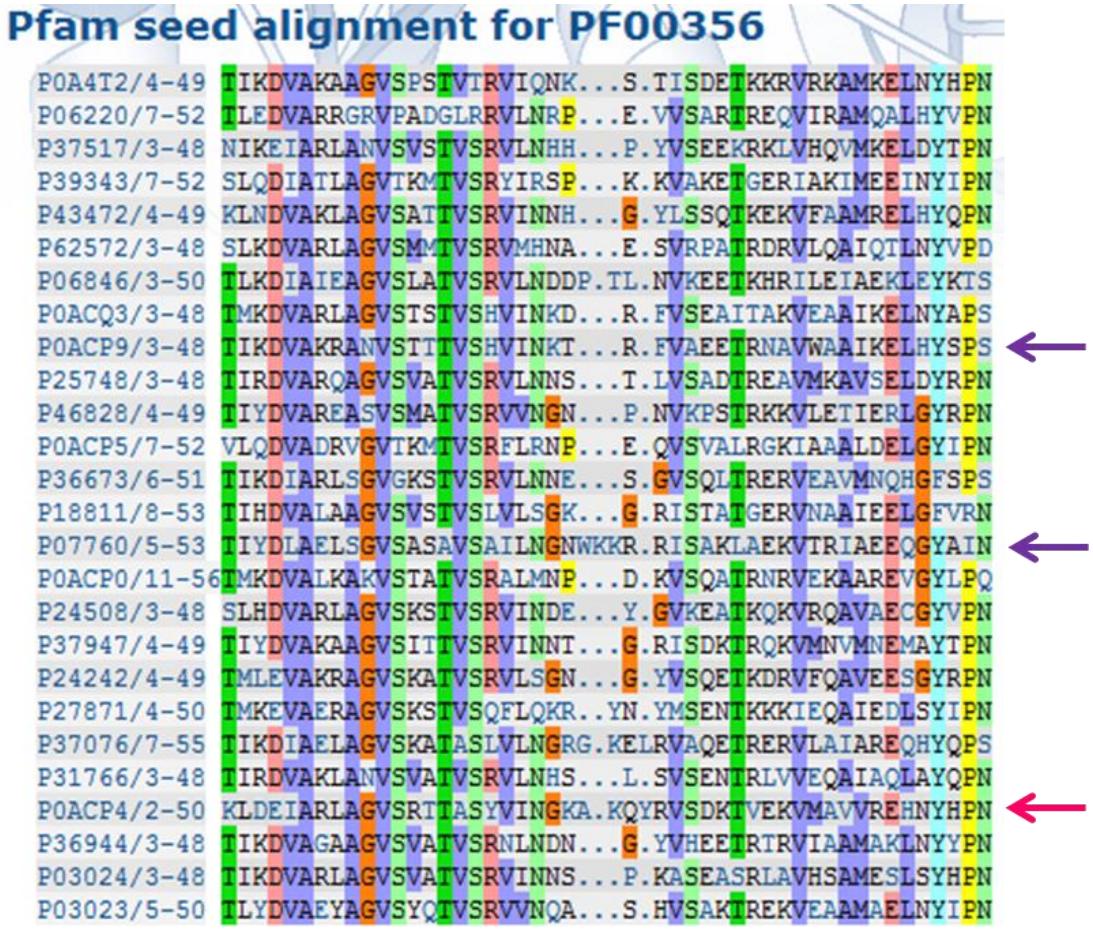


Figure 4.1: LacI-GalR pfam alignment for FruR with arrows indicating sequences which contain a Trp (Petrik)

We also needed this Trp residue to be buried within the hydrophobic core of the protein, when folded, and exposed to the solution, when unfolded. This addition will allow us to observe the folded and unfolded states *via* fluorescence. This goal we knew would be very difficult to achieve due the small size of FruR and because it has a very small core. Trp is a relatively large residue so integrating it within FruR will be a challenge. Y19W and Y28W were chosen because they each contained tyrosine which is a large hydrophobic residue similar to tyrosine. We also picked these positions because

they contribute to the stabilization of the molecule due to stacking of the aromatic rings and formation of hydrogen bonds between them[6]. This is shown in Figure 4.2 below. Based on the alanine scan, these specific mutations were chosen: 12W, 38W, and 6W which showed the residues within FruR are especially important towards the specific folding of WT FruR. We also created these mutations virtually in Yasara[24] and tested them using FoldX[24] to see if any steric clashes would occur.

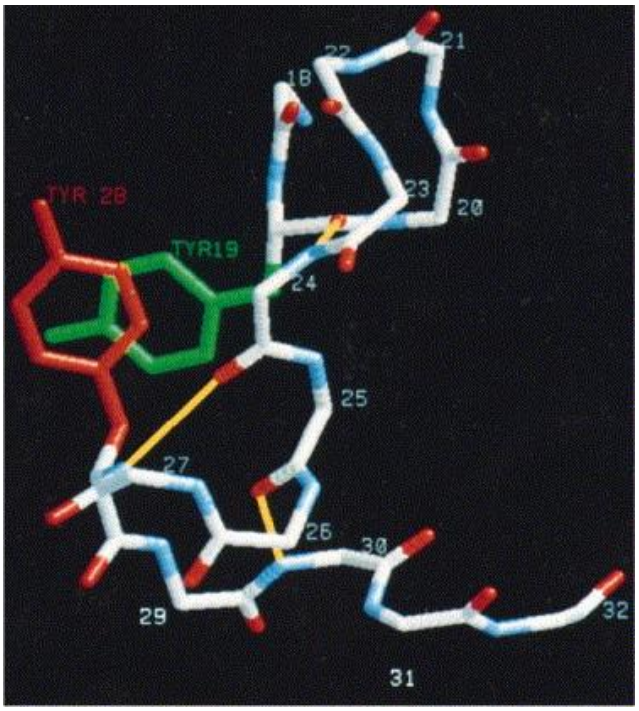


Figure 4.2: Shows stability by stacking and H-bonds between Tyr19 and Tyr28 of WT FruR [6].

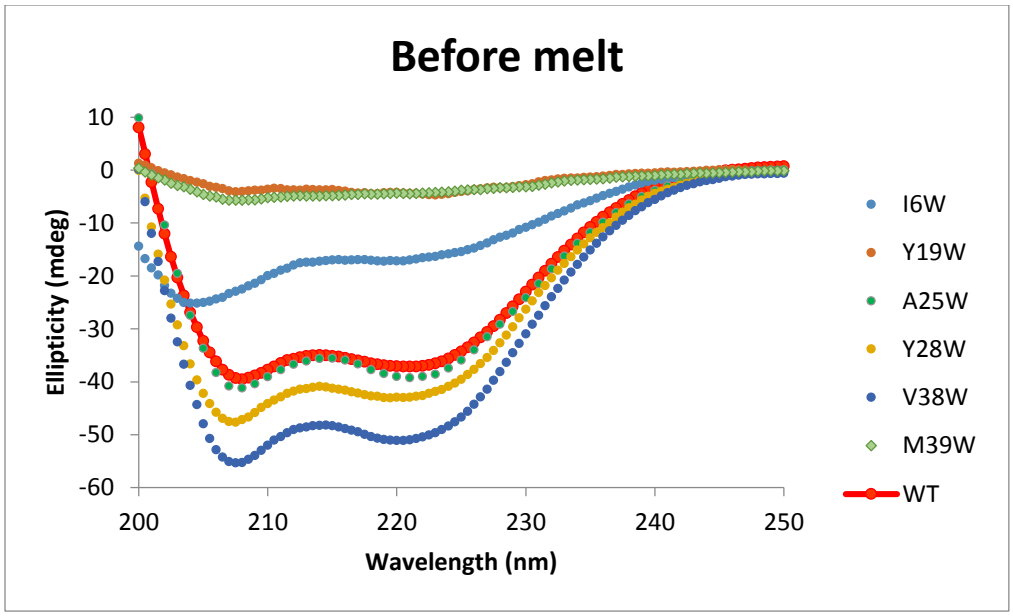


Figure 4.3: Before melt CD scan of WT FruR and Trp variants at 25°C

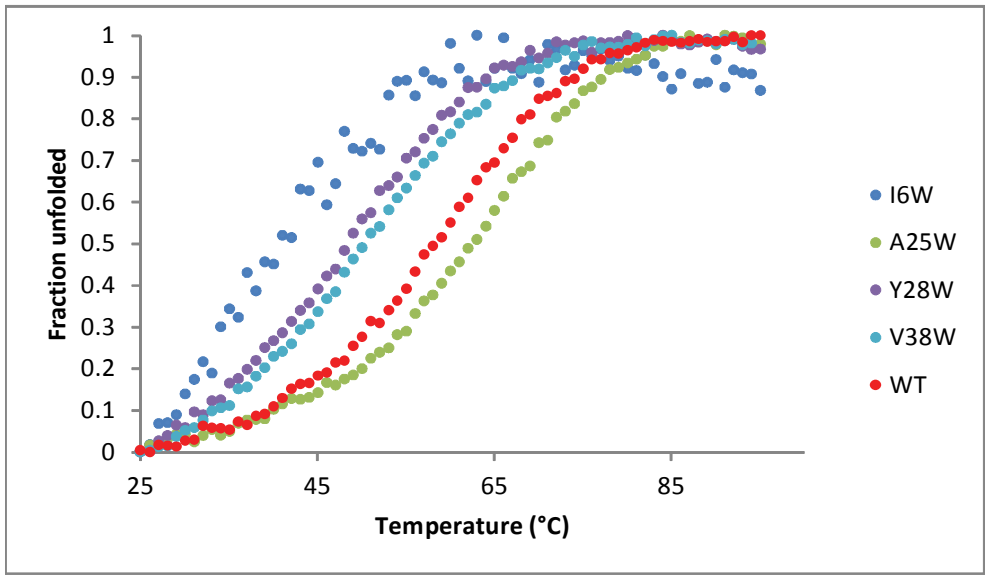


Figure 4.4: Thermal Melt Scan of WT FruR and variants

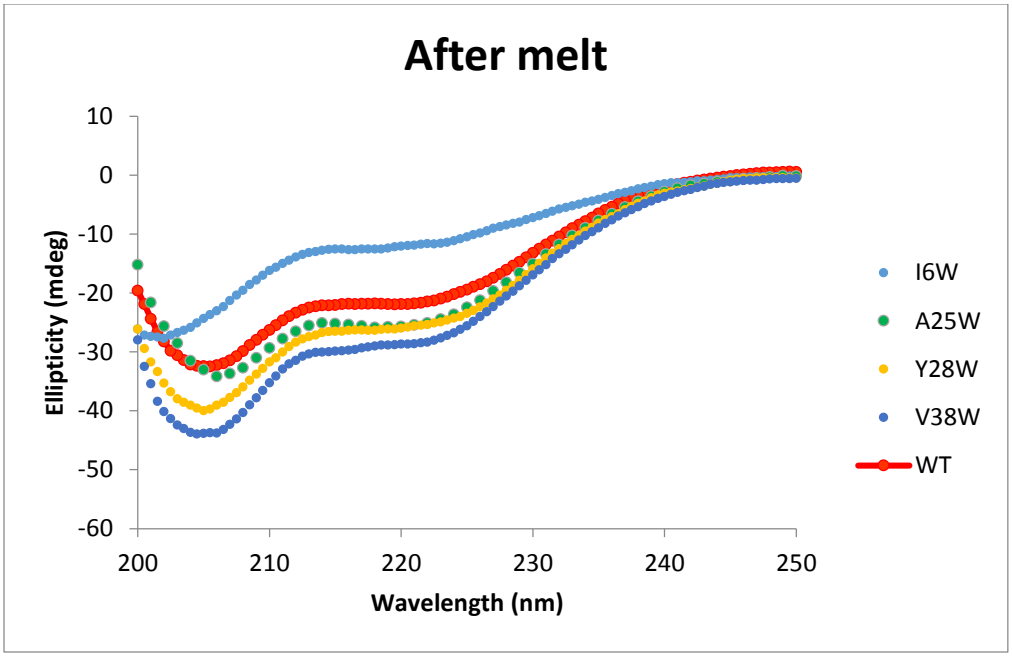


Figure 4.5: After melt CD scan taken at 25 °C to see if folding is reversible

These CD scan results show which specific mutants fold like WT FruR which is predominantly alpha helical with significant peaks at 208 nm and 222 nm. V38W, Y28W, and A25W all were shown to fold like WT, while I6W, Y19W, and M39W were unfolded or molten globule. So, we continued our studies with V38W, Y28W, and A25W. Our next step was to do a thermal melt *via* CD machine in order to analyze the stability of these mutations in comparison to WT FruR where we heat up the protein samples to a maximum of 90 °C. We then fit the data to a Gaussian curve in order to observe the melting temperatures more easily. These data are shown above in Figure 4.4, which displays the mutant's stability by melting temperature in comparison to WT FruR. We then took another CD scan of the protein after cooling back down to 25 °C shown in Figure 4.5.

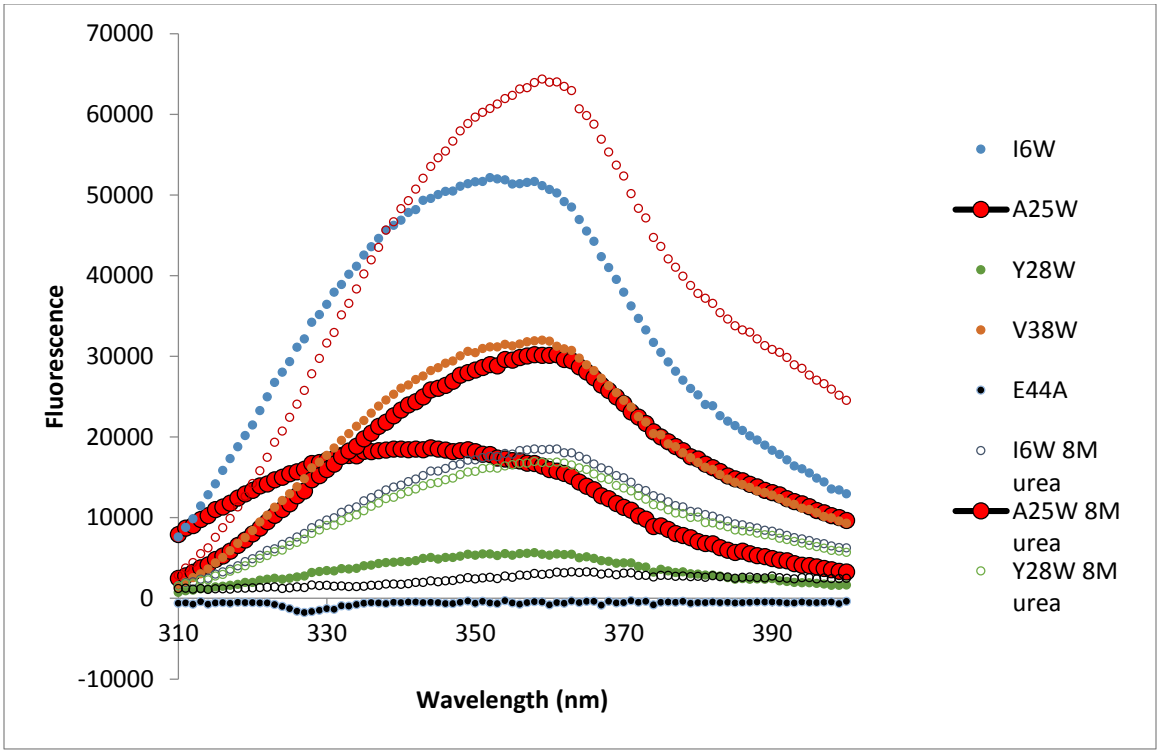


Figure 4.6: Fluorescence of folded and unfolded Trp variants

These results are particularly interesting because one of the variants, A25W, is shown to be more thermodynamically stable than that of the WT structure. This is quite surprising considering that this variant includes the addition of a large tryptophan residue within FruR's very small hydrophobic core. We can now measure the fluorescence of these variants and conclude whether we have indeed obtained a successful fluorescent mutant. This data are shown in Figure 4.6 above.

Table 4.1 below shows that all the Trp mutants are less stable than WT except for A25W. A25W turned out to be more stabilizing than WT by 5 °C. These data show that we indeed introduced a fluorescent residue within the hydrophobic core of the molecule. We took a scan of both the folded protein in CD buffer and the unfolded protein with the addition 8 M Urea, where the protein is unfolded.

These fluorimeter results show that I6W and Y28W either are already unfolded prior to the addition of 8 M urea or the Trp residue is displayed on the surface of the protein and not within the hydrophobic core of the molecule, therefore making it impossible to tell the difference between the folded and unfolded states. Together with the CD data indicates that these variants were unfolded. However, A25W (folded shown in red and unfolded shown in purple) successfully has its Trp residue buried within the hydrophobic core of the molecule, showing a broad peak between 330 nm and 360 nm. The unfolded A25W variant shows a higher intensity of fluorescence due to the Trp being exposed and has a significant peak at 360 nm. This will make further characterization of the intermediate variants when flipping the fold from FruR to Er23 much easier to visualize and characterize in future experiments. High throughput methods can now be used to analyze FruR and intermediate variants by using 96 or 384 well plates for analysis using small volumes (10-20 µL) of the protein (Figure 4.7).

				CD		Fluorescence	
Variannt	PCR	Seq	Expr	scan	Tm	NaPi	8Murea
I6W	✓	✓	✓	✓	MG	355	360
A25W	✓	✓	✓	✓	60	~345	360
Y28W	✓	✓	✓	✓	44.5	360	360
V38W	✓	✓	✓	✓	46.5	360	360
M89W	✓	✓	✓	✓	MG		

Table 4.1: Summary of Trp variants made and what information was collected from each (MG stands for molten globule)

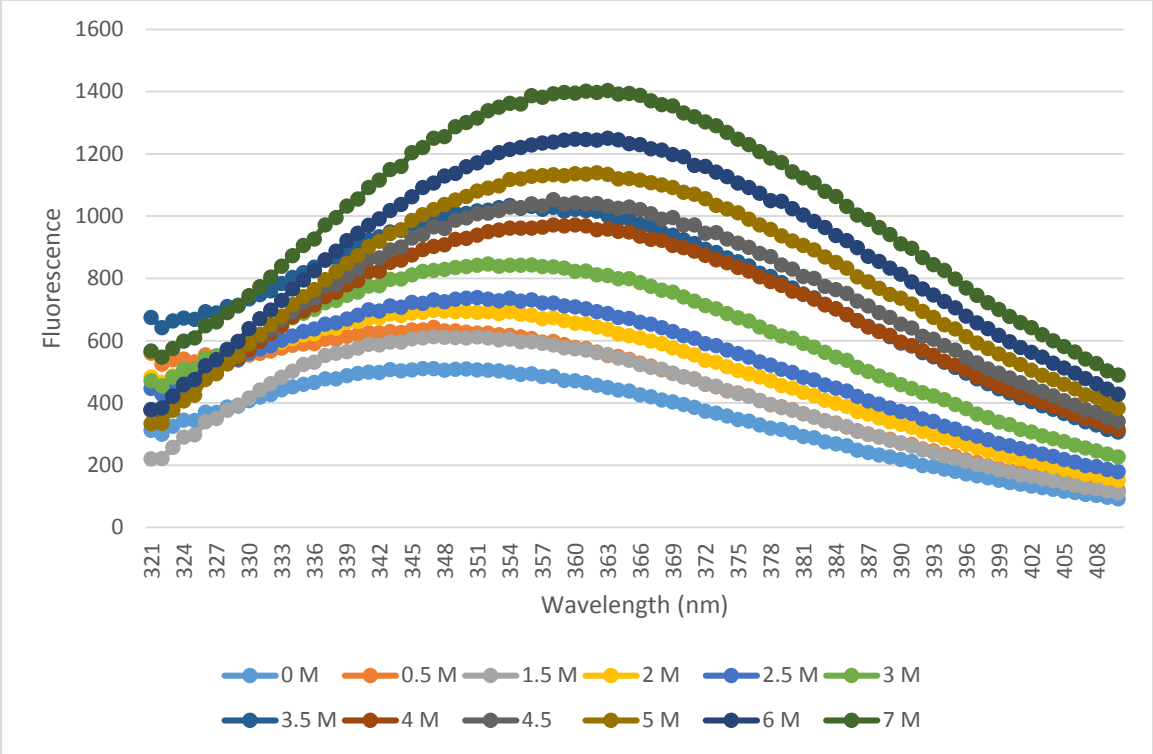


Figure 4.7: HTTS: 384 well plate urea melt of FruR A25W variant

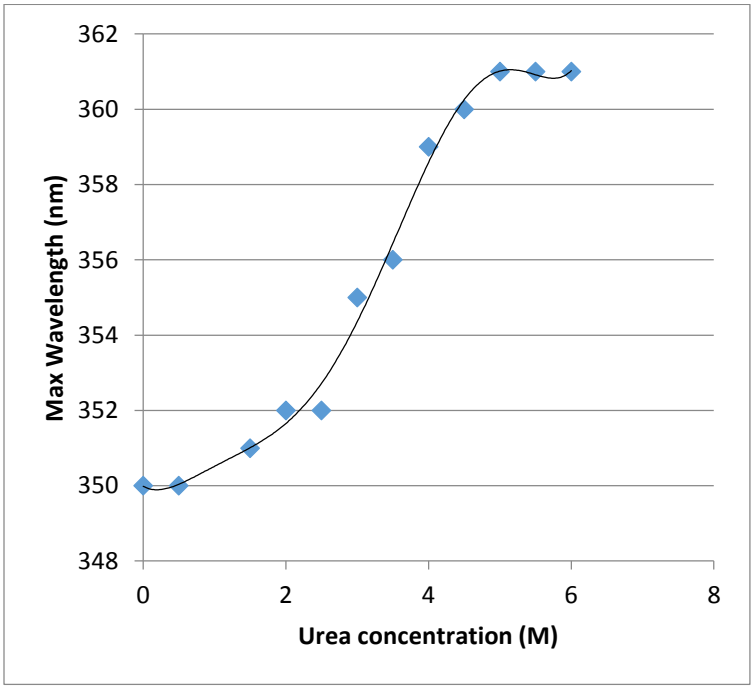


Figure 4.8: A25W graphed analysis of the shift in the peak with change in urea concentration.

Figure 4.8 above is a fluorescence scan of a urea melt of the successful A25W variant of FruR to see if expression could be scaled down to 20 µL while still getting a strong enough signal. This data proves that high-throughput methods can indeed be used in future characterization of this protein. The graph below this shows how the fluorescent peak changes with increasing urea concentration. As the protein unfolds the peak shifts to a higher wavelength signifying that the fluorescent Trp residue is becoming more exposed to solvent thus making the intensity of fluorescence increase. This data was collected by Dr. Eva Petrik.

4.2: Synthesis and Characterization of WT Er23

When we set out to express WT Er23 we knew it would be difficult due to the large number of disulfide bonds within the structure of the molecule. Er23 contains 10 cysteine residues which make 5 disulfide bridges within the protein. Because we knew these Cys residues would make this protein particularly difficult to express and purify we decided to consider two ways in which we could express this protein: by periplasmic and cytoplasmic expression methods. Cytoplasmic expression is the usual way in which proteins in the Magliery lab are expressed and it is also the method by which FruR was expressed. Periplasmic expression is used mainly for proteins like Er23 with multiple disulfide bonds. This expression method is useful because it helps to fold the disulfide bonds by creating an oxidizing environment, also contains two foldases, DsbA and DsbC, which catalyze formation and isomerization of disulfide bonds [22].

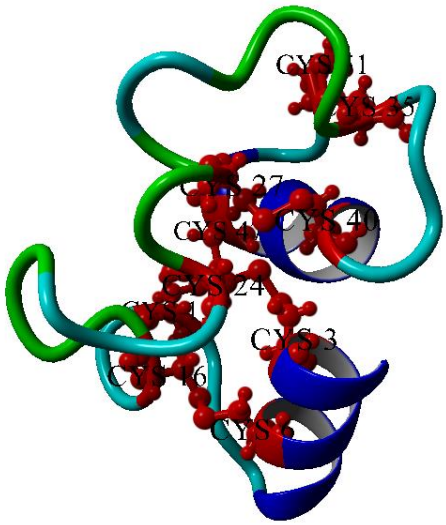


Figure 4.9: WT Er23 with all disulfide bonds shown in red. Made using Foldx[24]

Although we had these methods planned, we had Er23 already cloned into Origami B cells by Venuka Durani[25] and had successfully expressed using cytoplasmic method. So, we used this method for further expression of Er23. In addition to using regular methods of expression we also wanted to see if we could express this protein using minimal media in order to obtain an NMR scan of Er23.

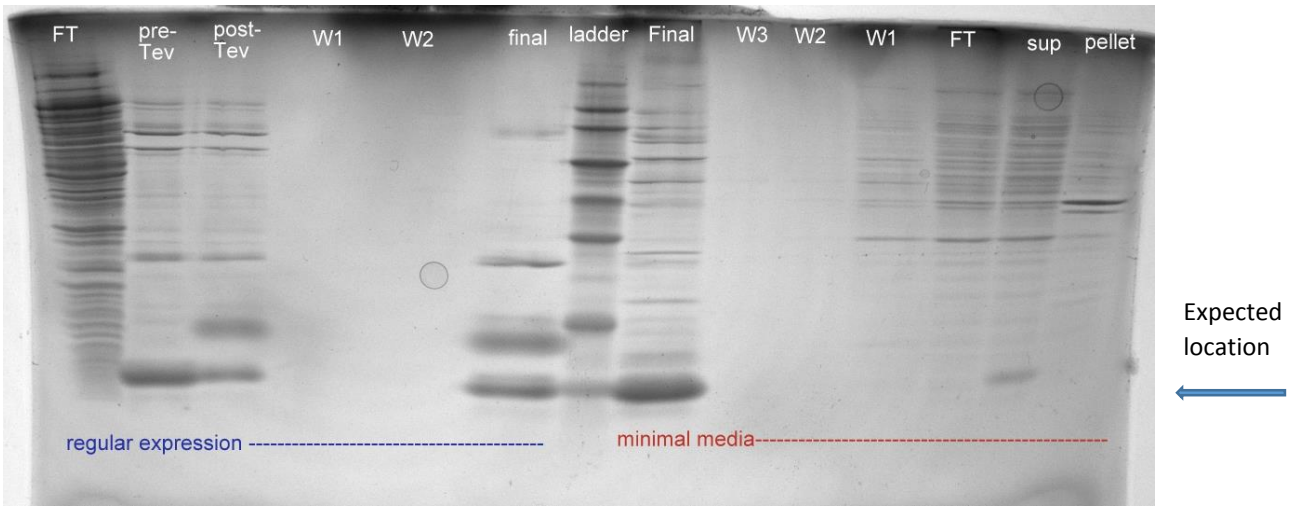


Figure 4.10: SDS-Page gel showing expression of Er23 by regular and minimal media

Once expressed we wanted to characterize this protein by many methods including NMR, mass spec, CD, and guanidine melts, crystallography, etc. The ^{1}H NMR spectrum below shows well dispersed peaks signifying a well folded protein structure.

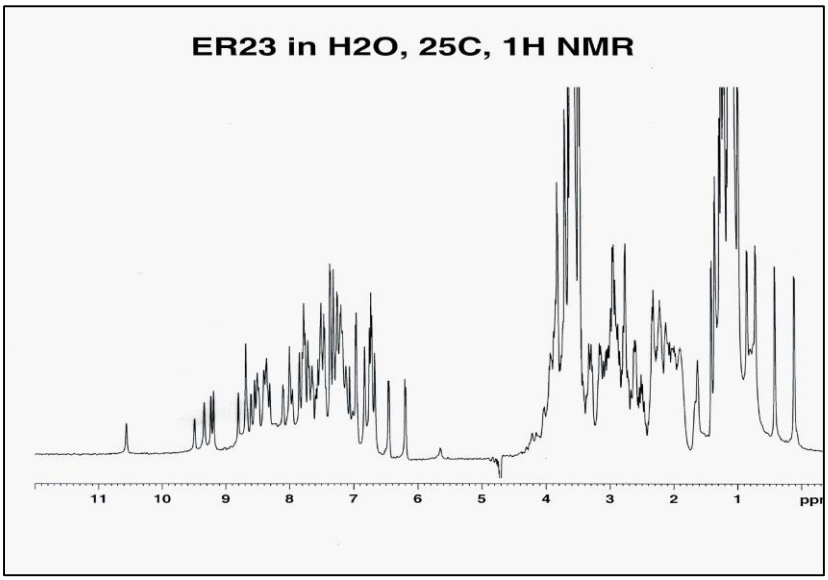


Figure 4.11: Proton NMR of WT Er23

The mass spectrum shown below shows the expected mass for Er23 which once again signifies that we do have the correct WT Er23 structure.

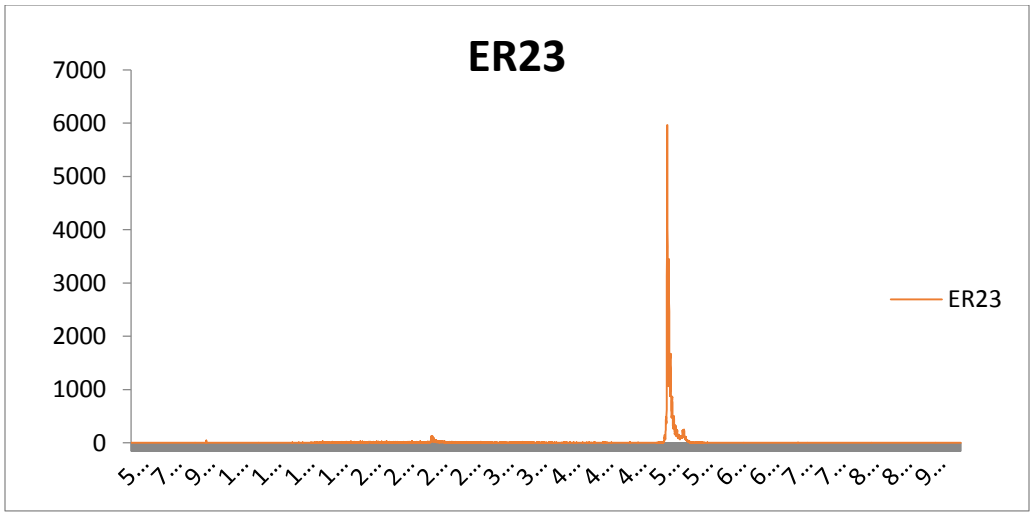


Figure 4.12: Mass Spectrometry of Er23 (mass is 5104 Da)

We first wanted to conduct an Ellman Reaction in order to see if the disulfide bonds within the molecule were folded correctly within the structure. Only with the addition of a reducing agent, TCEP, would we be able to see a change in color of the sample to yellow, indicating the existence of free thiols. Only samples which contained TCEP turned yellow with addition of Ellman Reagent. When subtracting the background from the samples, there was a negligible difference even with addition of reducing agent there are still no free thiols being exposed. This concluded that the disulfide bonds within Er23 were indeed folded.

The CD spectra results below show a significant peak at 209 nm for WT Er23 which is expected for a protein which has disulfide bonds and alpha-helices[23]. After heating the molecule up and doing a thermal melt another scan was taken. There were no differences between the scans taken before and after the stability melt which signifies a very stable protein. This we hypothesized was due to the tightly packed core of Er23 and many disulfide bridges present within its structure.

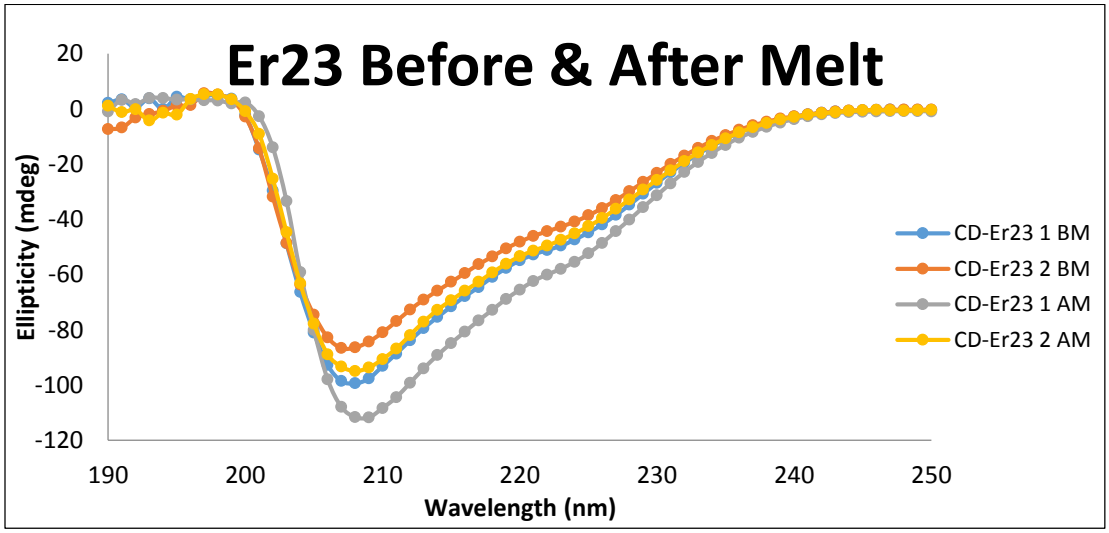


Figure 4.13: CD spectra of WT Er23 before melt and after melt scans taken to see is folding is reversible

This thermal melt data tracks the signal 222 nm while the molecule is heated up to 95 °C. If there was a difference in the structure while heating, the signal would either increase or decrease and this data shows no structural change because the data doesn't change from the scan taken before the melt to the scan taken after the melt was completed. This signifies a very stable molecule.

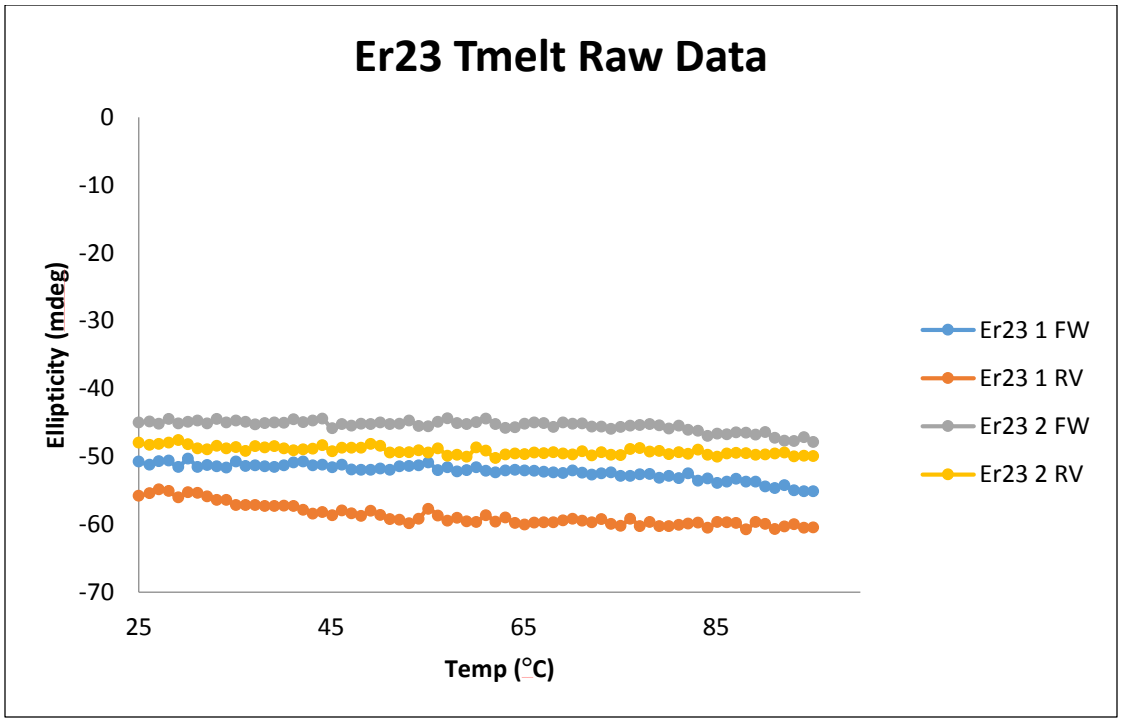


Figure 4.14: Thermal melt CD spectra of WT Er23

This CD data shows the extreme stability of WT Er23. Even when heating this protein to temperatures of 95 °C, the structure remains intact. This is shown in the thermal melt scan and shown in the CD scan above where the before and after melt scans look identical to one another. Because this data was so interesting we decided to take a closer look at the structure and stability of Er23 and to gain more information about disulfide bonds and how they contribute to stabilization and folding. In order to get a better understanding of how this protein folds and how stable the molecule is we decided

to conduct a urea melt of the protein by adding urea (9 M) with and without the addition of TCEP, a disulfide bond reducer to see if this would unfold the protein. The before melt scan shows that the protein is still relatively folded even with the addition of urea and TCEP shown in Figure 4.15 below.

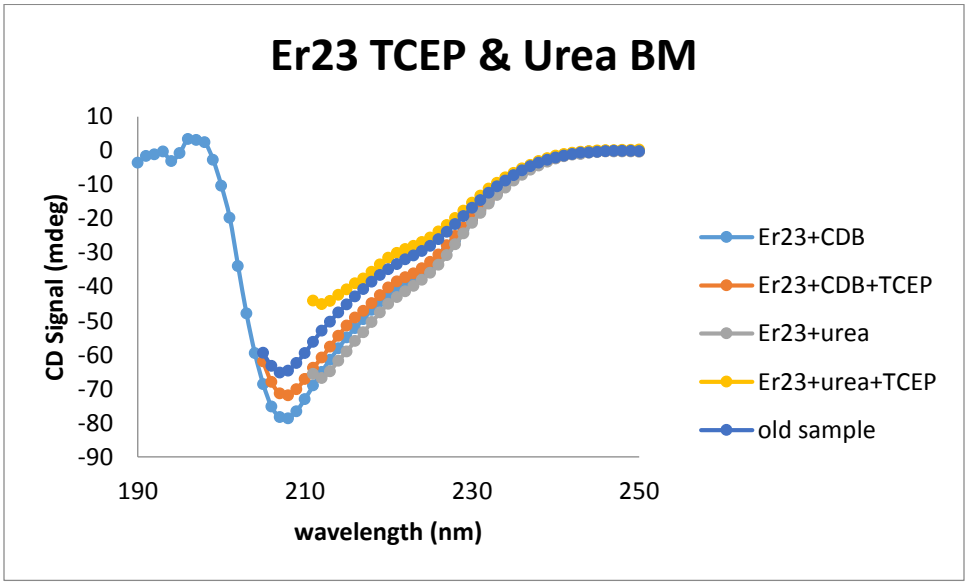


Figure 4.15: Before melt CD scans of Er23 with and without the addition of Urea and TCEP

These thermal melt scans shown below in Figure 4.16 depict that there is no significant change in the case of only adding urea to the protein. Only with the addition of TCEP and urea, the disulfide bond reducer, does the structure of the molecule begin to break apart and unfold. With the addition of only TCEP to the samples the structure does begin to unfold while heating shown in the gray and light blue data and is completely unfolded during the reverse scan shown in pink and gold.

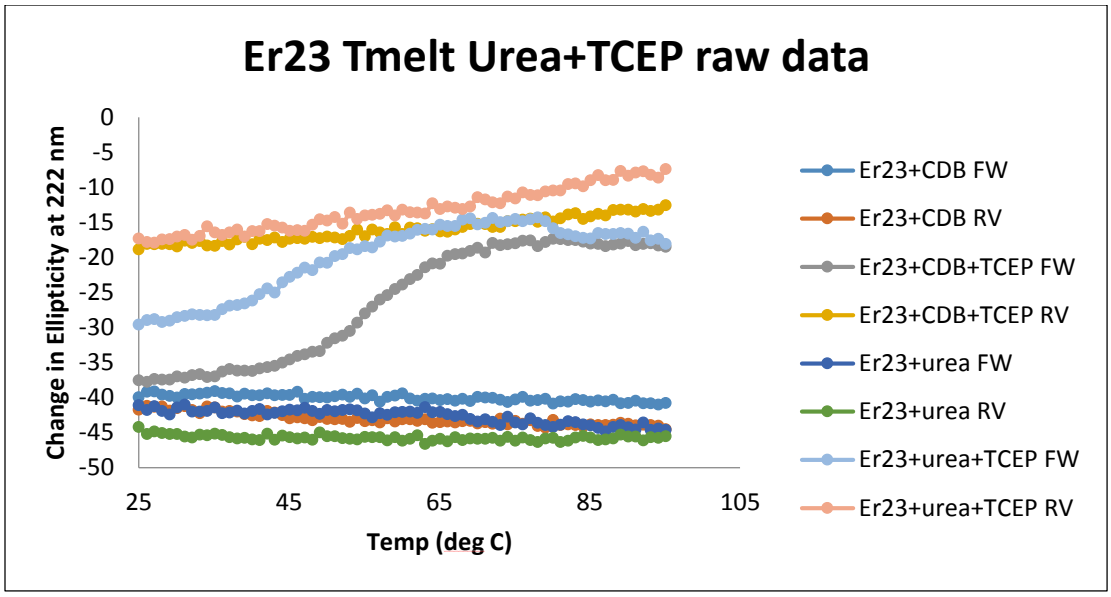


Figure 4.16: Thermal melt scans of urea melt

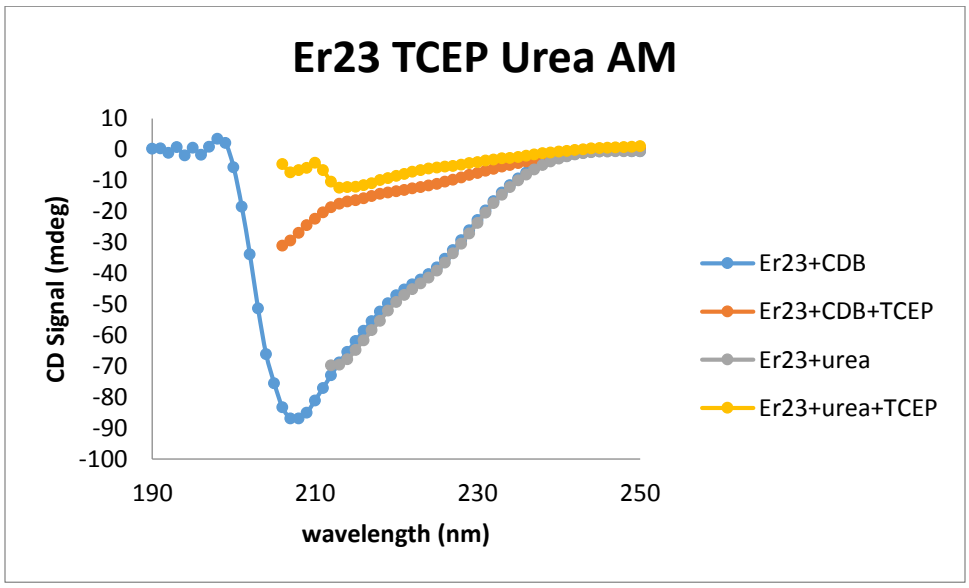
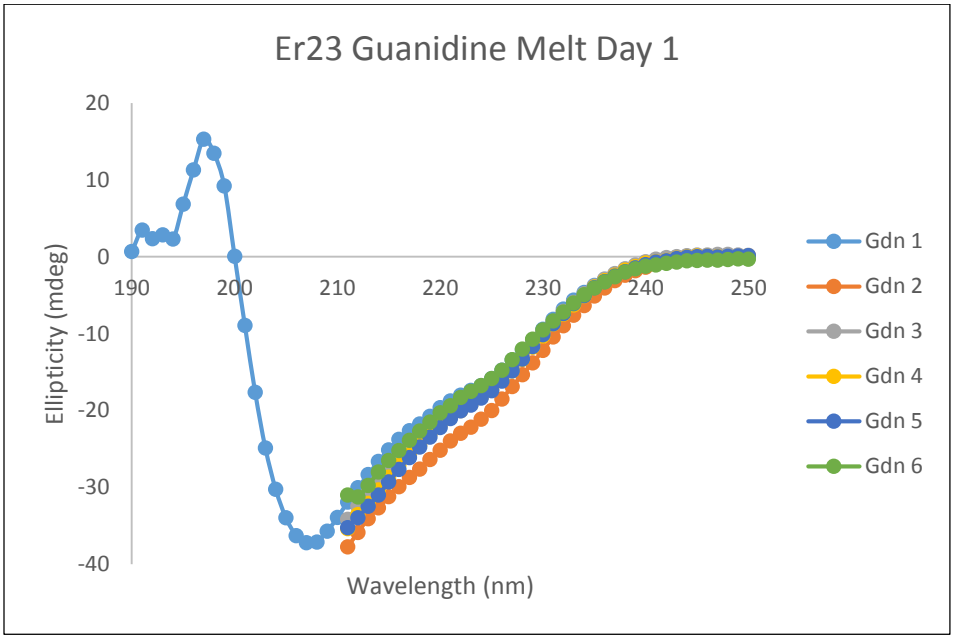


Figure 4.17: After melt CD scans of Er23 with and without the addition of urea and TCEP

This CD data taken after cooling the protein down to 25 °C, shows that in order to completely unfold Er23 you must add a disulfide bond reducer, such as TCEP, to break the disulfide bonds and degrade the protein by adding a denaturation agent such as urea and heat it up to unfold the structure completely.

Because Er23 was not unfolding completely with the addition of 8 M urea, we decided to conduct some more stability experiments of Er23 by using a different denaturation agent, guanidine also with and without the addition of TCEP by adding different concentrations of guanidine (from 0 M-5 M) and taking scans over 3 days. The data below in Figures 4.18 and 4.19 shows that after the initial addition of guanidine with and without TCEP, Er23 remains folded. Some of the data points are not shown due to an increase Dynovoltage over 600 V.



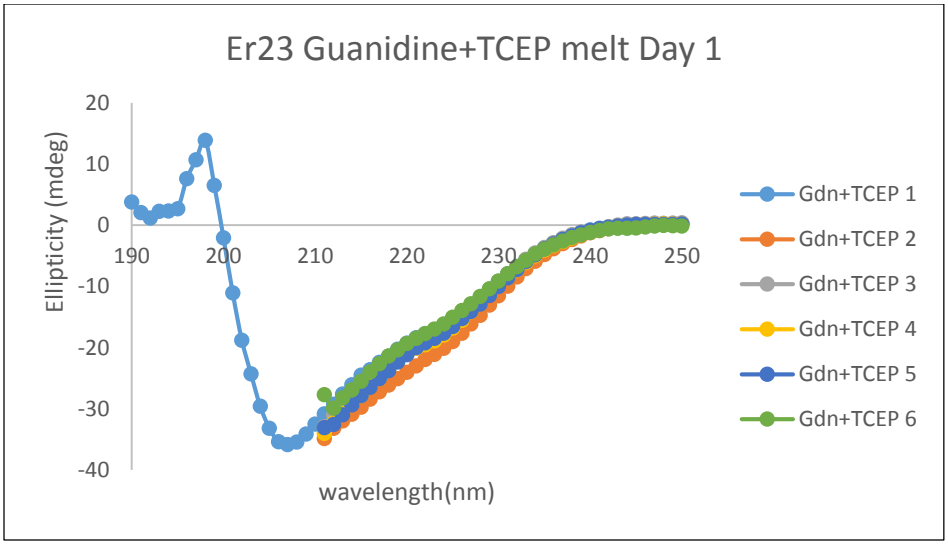
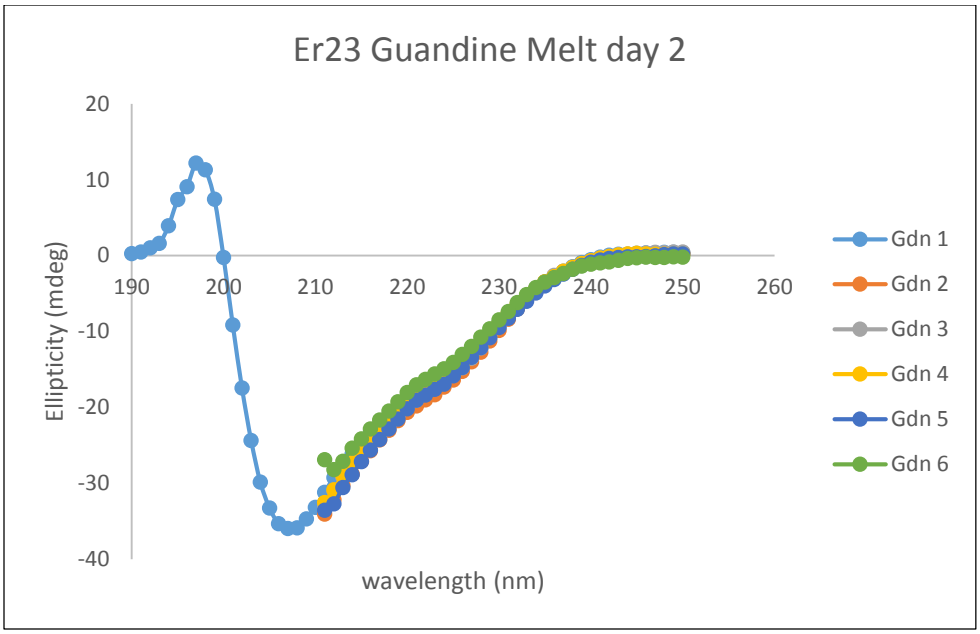


Figure 4.18 and 4.19: Day 1 CD scans of Er23 from 0 M to 5 M guanidine with and without addition of TCEP

After a 24 hour incubation in guanidine, the protein still is shown to be relatively folded shown in Figure 4.20. However, the samples which were incubated with the addition of TCEP, are beginning to unfold shown in Figure 4.21.



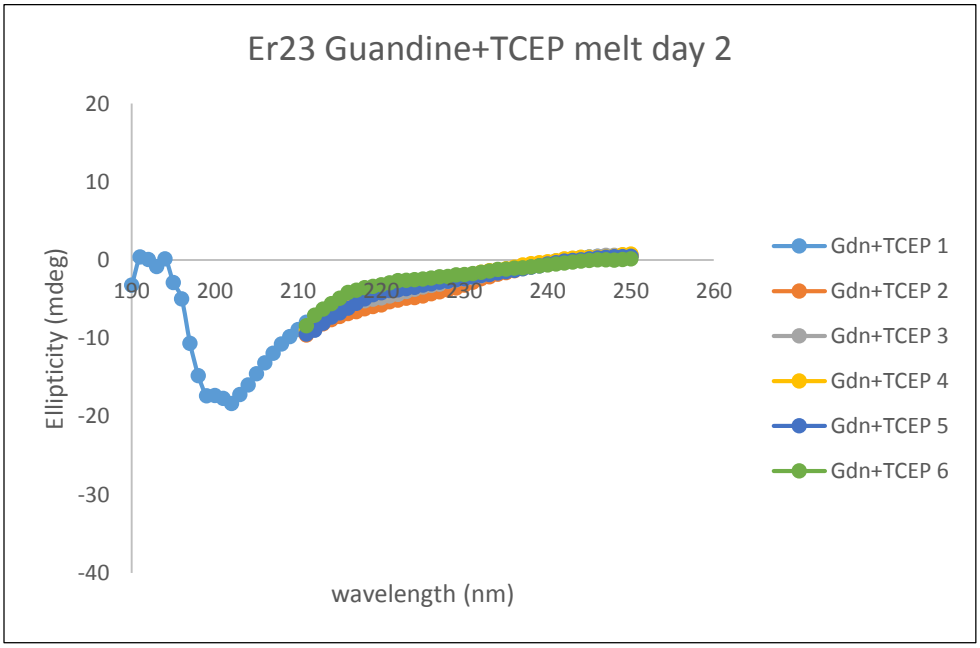


Figure 4.20 and 4.21: 24 hr incubation CD scans of Er23 from 0 M to 5 M Guanidine with and without addition of TCEP

Finally after 48 hour incubation in Guanidine with the addition of TCEP, the protein was shown to be completely unfolded. However, with only the addition of Guanidine, the protein is still remaining completely folded after 48 hours shown in Figure 4.22.

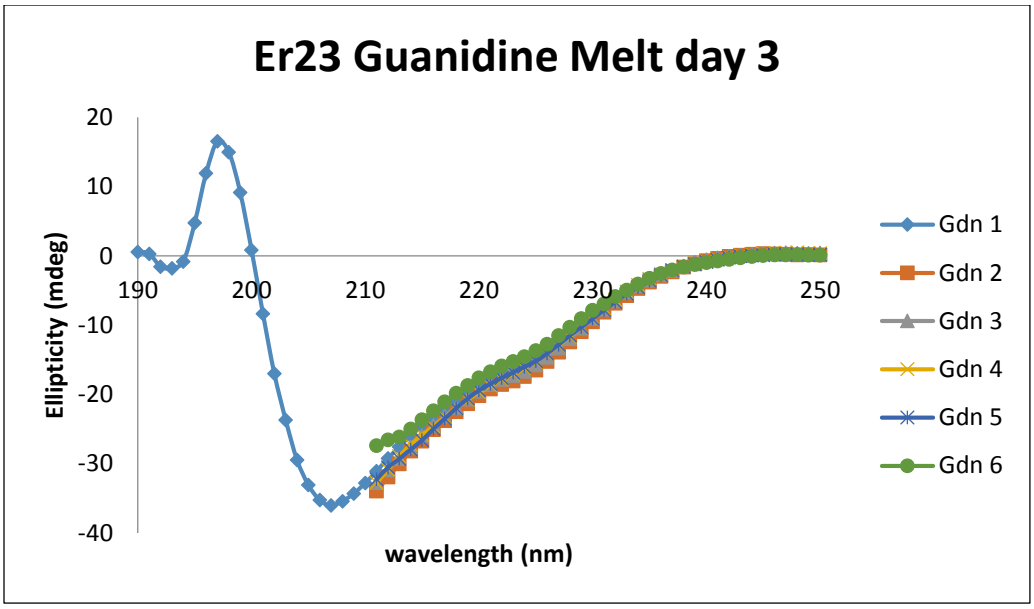


Figure 4.22: 48 hr incubation CD scans of Er23 from 0 M to 5 M Guanidine

The first and second day data show that not much unfolding has occurred with the addition of guanidine. After the third day, Er23 was completely unfolded with the addition of TCEP; however, with just the addition of guanidine had no effect on the structure of Er23.

We also wanted to set up a series of crystallography conditions to try and obtain a crystal structure of Er23. So far, we have gotten some promising results from these specific conditions.

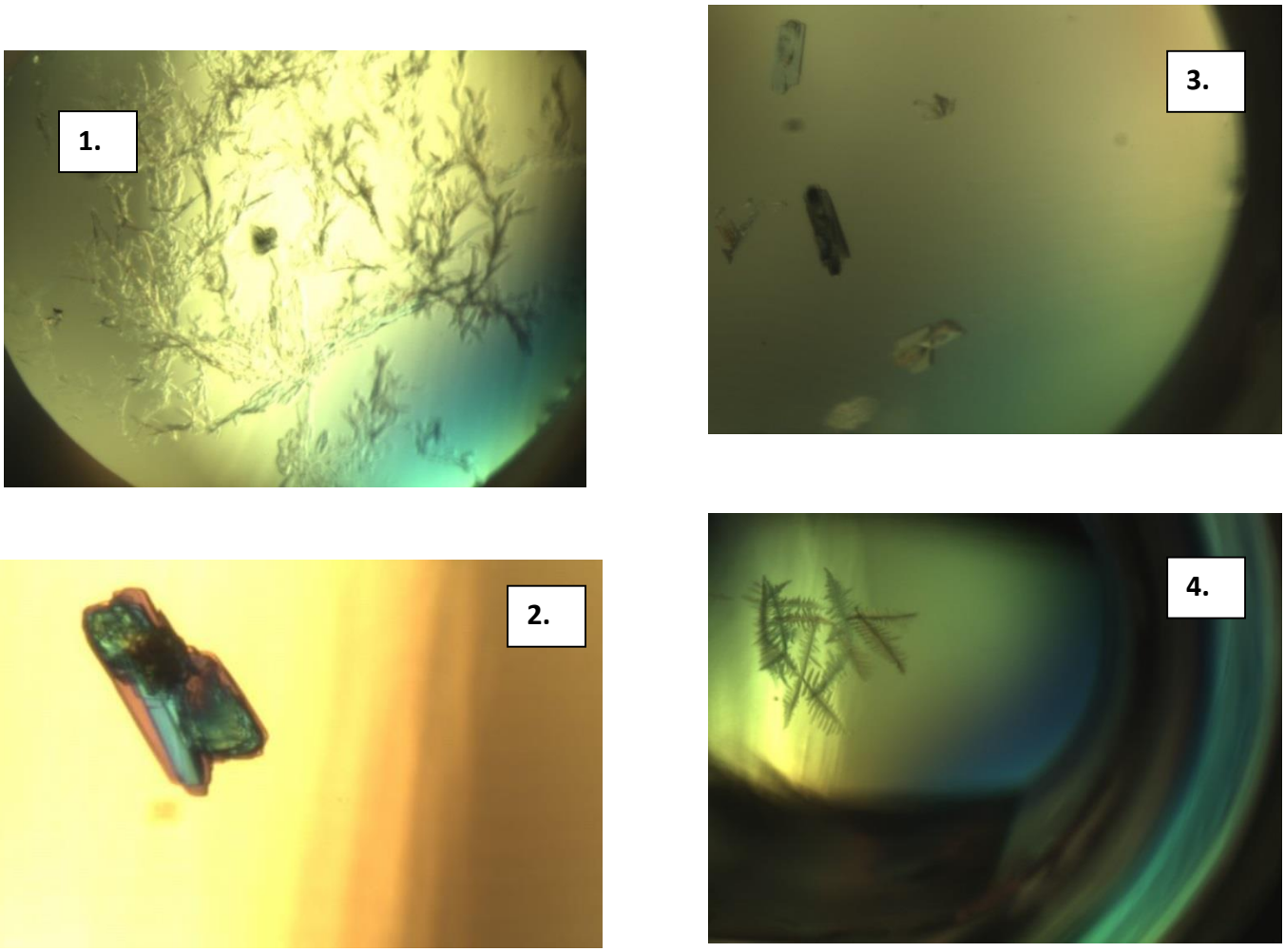


Figure 4.23: Crystallography of Er23 1. 0.1 M Tris, 3.4 M 1,6 hexanediol, 0.2 M magnesium chloride; 2. O.1 M bicine pH 9, 2 M magnesium chloride; 3. 0.1 M Sodium Acetate , pH 4.6, 20%, Isopropanol, 0.2 M calcium chloride; 4. 0.1 M TRIS, pH 8.5, 30% Isopropanol, 0.2 M Ammonium Acetate

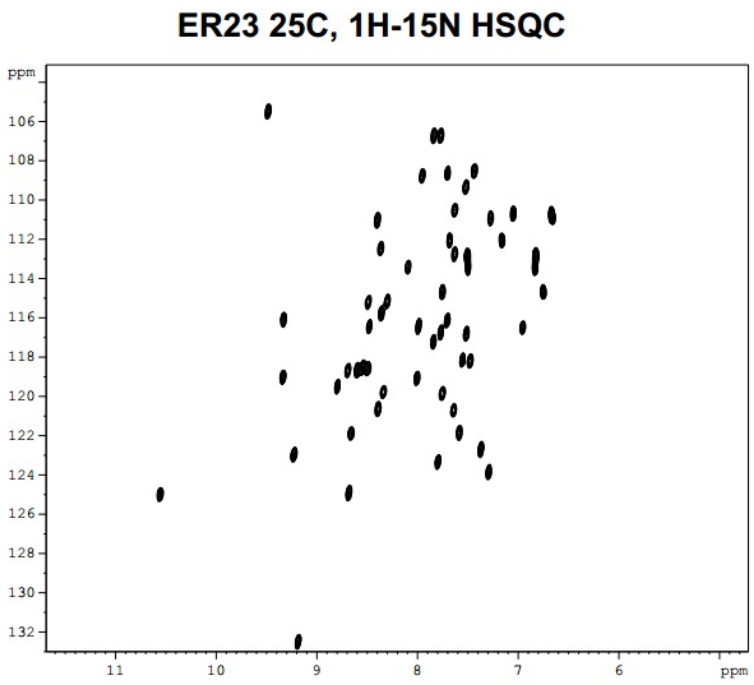


Figure 4.24: HSQC of WT Er23

Lastly, we decided to obtain an HSQC NMR of Er23 in order to give us a better idea of what the structure looks like. This HSQC data shows us not only that WT Er23 is definitely folded, but also will allow us to solve the structure of Er23 and compare this with the Cys-free variants in the future. We can deduce that the structure is folded because there are about 50 cross-peaks which are well defined and sharp.

4.3 Synthesis and Characterization of Er23 Cys-free variants

When deciding which mutations to make to WT Er23 we knew that we wanted to start by individually taking out each disulfide bond and replacing them with Ala-Val and Val-Ala mutations which has been shown to work with Rop protein within the Magliery lab[18]. This is because not only did we want to begin the flipping fold experiment, but another goal of ours was to see the stability effects of disulfide bonds on Er23. Also, the

main difference between FruR and Er23 is the addition of disulfide bonds within the structure of Er23 and lacking in the structure of FruR. In order to predict the destabilizing effects of these mutations we made them computationally within FoldX[24] and used this program to measure the stability by ΔΔG. Those variants which had the smallest ΔΔG were the variants which were most similar to WT Er23 in stability.

ΔΔG	**Ala-Val**	**Val-Ala**
Cys 35&51	3.14	2.93
Cys 13&47	3.92	2.5
Cys 3&24	5.52	1.79
Cys 27&40	2.55	4.45
Cys 6&16	0.6	2.57

Table 4.2: shows the ΔΔG's of the specific Cys-free mutants analyzed by Foldx[24].

Based on the ΔΔG data we collected, we decided to move forward and mutate the disulfide bonds with cysteine residues at 6&16 and 3&24. We wanted to try to replace them with both Ala-Val and Val-Ala mutations and analyze the stability effects of these mutations in comparison to WT. Once sequences for variants 1: 6&16 Ala-Val, 2: 6&16 Val-Ala, 3: 3&24 Ala-Val were confirmed we expressed and purified them.

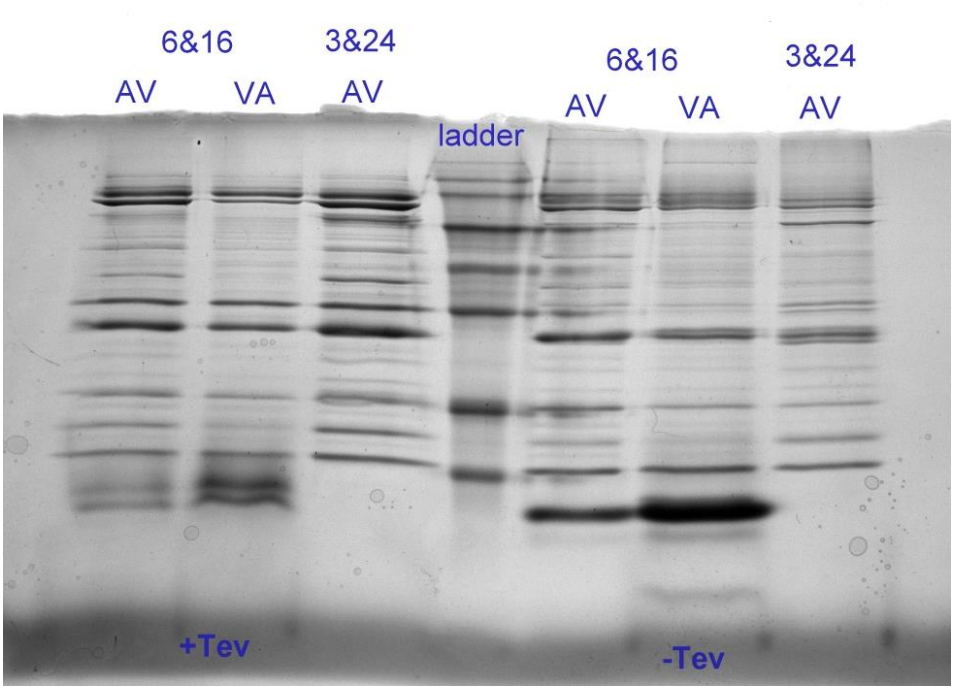


Figure 4.25: SDS-Page purification gel showing Tev reaction of variants 1, 2, and 3 (Petrik)

As shown in Figure 4.25 above, the expression of both variants of 6&16 Ala-Val and Val-Ala were successful. However, variant 6&16 Ala-Val expression failed. This was possibly due to the mutation of the disulfide bond at this position. This specific disulfide bond may be needed in order for Er23 to fold correctly and successfully express.

Soon, we also got the last variant 3&24 Val-Ala sequence confirmed and expressed this along with a second attempt at expression of the 3&24 Ala-Val variant. This second attempt and first attempt at expression of these variants also failed. This further led us to the conclusion that the removal of this disulfide bond is detrimental to the folding of Er23 and caused the protein to unfold.

Chapter 5

Discussion and Future Work

A successful variant of FruR has been synthesized and purified containing an introduction of a Trp residue within the hydrophobic core of the protein. This variant will make further characterization of mutants for the flipping fold experiment easier due to the use of high throughput methods.

Wild type Er23 has been successfully expressed and has been shown to be folded and to have disulfide bonds within its structure correctly formed. Further characterization of this protein is to be conducted in the future including the production of a solved crystal structure.

Synthesis of the first four Cys-free variants has been conducted and characterization of these mutants is to be done in the future. It has been shown that variants 6&16 Ala-Val, 6&16 Val-Ala, and 3&24 Ala-Val all successfully express. Whereas, variant 3&24 Val-Ala does not successfully express. This could be due to the removal of a crucial disulfide bond for the correct folding of Er23. These mutants will be initial studies towards the flipping of Er23 into FruR. They will also give insight into the stabilizing effects of removing disulfide bonds.

Future work includes further characterization of wild-type Er23 as well as expression and characterization of Er23 Cys-free variants. Another possible direction for the flipping fold experiment could be to introduce disulfide bonds into the structure of FruR and see the effects of these mutations on the structure.

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